Analysis of aspects of starch metabolism in *Physcomitrella patens*

by

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Declaration

By submitting this dissertation electronically, I the undersigned, hereby declare that the work contained therein is my own, original work (Unless to the extent explicitly otherwise stated) and that I have not previously, in its entirety or in part submitted it to any university for a degree.

Signed……..…….. Date…….

Abstract

Starch is an important polysaccharide produced by plants and is widely used in industry mainly as a food thickener, but also in other important processes, such as the development of textiles and paper manufacture. This polyglucan consists of two glucose polymers, amylose and amylopectin. As the major storage carbohydrate, starch is synthesised during the day before being catabolised at night to sustain plant growth and metabolism. Starch metabolism is well studied in vascular plants such as *Arabidopsis thaliana* and *Solanum tuberosum*, however, information about these processes are less well understood in non-vascular plants.

The bryophyte *Physcomitrella patens* is an excellent plant model system for many reasons; for example its fully sequenced genome and the ability to produce knockout mutants using homologous recombination. Attempts to gain a better understanding of the function and regulation of some of the key metabolic enzymes involved in starch metabolism in non-vascular plants have recently emerged (Stander, 2015; Jacobs, 2018; Mdodana et., al 2019**).** This project focused on two aspects of starch metabolism to determine whether some of the pathways and mechanisms involved during these processes are conserved between *Physcomitrella patens* and vascular plants.

The first part of this dissertation examines the roles of glucan water dikinase enzymes (GWD) in *P. patens*. In angiosperms these polypeptides are involved in starch degradation through catalysing starch phosphorylation. Five isoforms, PpGWDa-e, were identified in and phylogenetic analysis demonstrated the two (PpGWDa and PpGWDb) were most similar to *Arabidopsis* GWD1 or GWD2, while another two (PpGWDd and PpGWDe) were most similar to GWD3/PWD. The final isoform (PpGWDc) was likely to be inactive as it lacks the essential catalytic histidine. Both PpGWDa and PpGWDb targeted to chloroplasts. Using homologous recombination, knockout mutant lines were successfully generated for *PpGWDa* and *PpGWDb* each isoform both as individually and together in double mutants. Inserts in either gene resulted in reduced amounts of starch phosphate compared to the control, with *Ppgwda* mutant lines interestingly containing less glucose 6-phosphate in starch than *Ppgwdb* lines. Double mutant (*Ppgwda/Ppgwdb;* DM) lines contained even less glucose 6-phosphate in starch than *Ppgwda* single mutants. When plants were grown over a diurnal cycle it was shown that, like vascular plants, starch

accumulated in the light period and was degraded at night. Both *Ppgwd1a* and DM lines accumulated significantly higher amounts of starch compared to *Ppgwd1b* and the control lines at almost all time points. Soluble sugars on the other hand were significantly reduced in *Ppgwd1a* and the DM lines compared with *Ppgwd1b* and the control lines. The *Ppgwd1a* and DM mutant lines also demonstrated an observable morphological phenotypic alteration characterized by lack of gametophore development which could be reversed by growing the plant on media supplemented with glucose.

In the second experimental chapter, two isoamylase (ISA) like starch debranching enzymes, ISA1 and ISA2, were examined. Mutations in these genes in vascular plants can result in the production of the water-soluble polysaccharide (WSP), phytoglycogen at the expense of starch. Both single and double mutants showed loss of a debranching enzyme activity band in activity gels indicating that ISA1 and ISA2 exist as a heterocomplex in *Physcomitrella patens*. Analysis of purified recombinant protein demonstrated, surprisingly that both PpISA1 and PpISA2 peptides were catalytically active. Analysis of single and double mutant plants demonstrated that all contained increased amounts of WSP.

Opsomming

Stysel is 'n belangrike polisakkaried wat deur plante geproduseer word en word wyd gebruik in die industrie, hoofsaaklik as voedselverdikker, maar ook in ander belangrike prosesse, soos die ontwikkeling van tekstiele en papiervervaardiging. Hierdie poli-glukan bestaan uit twee glukose-polimere, amylose en amylopektien. As die belangrikste opbergkoolhidraat, word stysel gedurende die dag voordat dit in die nag gekataliseer word, gesintetiseer om plantgroei en metabolisme te handhaaf. Styselmetabolisme word goed bestudeer in vaskulêre plante soos Arabidopsis thaliana en Solanum tuberosum, maar inligting oor hierdie prosesse word minder goed verstaan by nie-vaskulêre plante.

Die bryofiet Physcomitrella patens is om baie redes 'n uitstekende plantmodelstelsel; byvoorbeeld die volledig volgorde genoom en die vermoë om uitklopmutante te produseer met behulp van homoloë rekombinasie. Pogings om 'n beter begrip te kry van die funksie en regulering van sommige van die belangrikste metaboliese ensieme wat betrokke is by styselmetabolisme by nie-vaskulêre plante, het onlangs na vore gekom (Stander, 2015; Jacobs, 2018; Mdodana et., al 2019). Hierdie projek het op twee aspekte van styselmetabolisme gefokus om te bepaal of sommige van die paaie en meganismes wat tydens hierdie prosesse betrokke is, tussen Physcomitrella patens en vaskulêre plante bewaar word.

Die eerste deel van hierdie proefskrif ondersoek die rolle van glukanwater-dikinase-ensieme (GWD) in P. patens. In angiosperme is hierdie polipeptiede betrokke by styselafbraak deur styselfosforylering te kataliseer. Vyf isoforme, PpGWDa-e, is in Physcomitrella patens geïdentifiseer. Filogenetiese analise het getoon dat die twee (PpGWDa en PpGWDb) die meeste ooreenstem met Arabidopsis GWD1 of GWD2, terwyl nog twee (PpGWDd en PpGWDe) die meeste ooreenstem met GWD3 / PWD. Die finale isoform (PpGWDc) was waarskynlik onaktief omdat dit nie die essensiële katalitiese histidien het nie. Beide PpGWDa en PpGWDb is gemik op chloroplaste. Met behulp van homoloë rekombinasie is uitklopmutante lyne suksesvol gegenereer vir PpGWDa en PpGWDb elk isoform, sowel as individueel en saam in dubbele mutante. Insetsels in een van die gene het gelei tot verminderde hoeveelhede styselfosfaat in

vergelyking met die kontrole, met Ppgwda-mutantlyne wat interessant genoeg glukose 6-fosfaat in stysel bevat as Ppgwdb-lyne. Dubbelmutante (Ppgwda / Ppgwdb; DM) lyne bevat nog minder glukose 6-fosfaat in stysel as Ppgwda enkelmutante. Toe plante oor 'n dagsiklus gekweek is, is dit aangetoon dat stysel, soos vaskulêre plante, in die ligperiode opgehoop het en snags afgebreek is. Beide Ppgwd1a- en DM-lyne het aansienlik hoër hoeveelhede stysel opgehoop in vergelyking met Ppgwd1b en die beheerlyne byna te alle tye. Aan die ander kant is oplosbare suikers aansienlik verminder in Ppgwd1a en die DM-lyne in vergelyking met Ppgwd1b en die beheerlyne. Die Ppgwd1a- en DM-mutantlyne het ook 'n waarneembare fenotipiese effek getoon, gekenmerk deur 'n gebrek aan gametofore-ontwikkeling wanneer dit op BCD-medium gekweek word. Dit kan omgekeer word deur die aanleg op media te laat groei, aangevul met glukose.

In die tweede eksperimentele hoofstuk is twee isoamylase (ISA), soos stysel-ontlas-ensieme, ISA1 en ISA2, ondersoek. Mutasies in hierdie gene in vaskulêre plante kan lei tot die produksie van die wateroplosbare polisakkaried (WSP), fitoglykogeen ten koste van stysel. Beide enkel- en dubbele mutante het die verlies van 'n debranching-ensiemaktiwiteitsband in aktiwiteitsgels getoon, wat daarop dui dat ISA1 en ISA2 as 'n heterokompleks in Physcomitrella patens bestaan. Analise van gesuiwerde rekombinante proteïen het getoon, verbasend dat beide PpISA1 en PpISA2 peptiede katalities aktief was. Analise van enkel- en dubbelmutante plante het getoon dat almal groter hoeveelhede WSP bevat.

Dedication

To God be the glory for the things he has done.

This publication is a dedication to my late niece, **Onako Mbilase**: you were deputy president of the Science Club for seniors at your school, you surely would have become a great scientist one day. Rest in perfect peace Mabhilita.

This publication is also dedicated to all the **young girls** whose lives were cut short as a result of gender-based violence in South Africa: Aluta continua!

And God shall wipe away all tears from their eyes, and there shall be no more death, neither sorrow, nor crying, neither shall there be any more pain.
Revelation 21: 4

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God Almighty: Ebenezer Nkosi yam, undigcinile kwaze kwabalapha, enkosi ngezibele zakho, iinceba zakho zingunaphakade. Thank you for believing in me and for pushing me to achieve more than I could ever have imagined. I praise you my Lord and indeed Let everything that has breath praise the Lord, **Psalm 150:6**; **Mat 3:16**. Amen.

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Chapter One

Literature review

1.1 Introduction

Starch is a homopolysaccharide formed by two distinct glucose polymers, amylose and amylopectin. These two polyglucans are structurally distinct and aggregate together leading to the formation of starch as semi-crystalline granules. Starch is synthesised in the plastids of land plants and many algal species where it functions as a carbon storage compound. Its presence appears almost universal in plants, with genomes from even the most primitive photosynthetic organisms containing genes that can be utilised to synthesise starch (Nougue et al., 2014). Indeed, a cyanobacterium has been demonstrated to synthesise a storage polysaccharide that shows some similarity to starch (Cenci et al. 2013), which implies that the evolution of the pathway leading to its synthesis may have pre-dated the endosymbiotic event that led to the development of plastids. The reason for its ubiquity within plants must be because its presence provides some fitness advantage and evidence produced over the past decade has shown that it is important for plant growth and, therefore, crop productivity (Graf and Smith 2011; Arias et al., 2014) as well as to abiotic stress resistance (Thalmann and Santelia, 2017). In addition to its role in plant metabolism, starch is also one of the most important products isolated from plants that is used by industry (Zeeman et al. 2010).

Because of its importance there has been much research into starch metabolism over the past century. Initial work concentrated on analysing mutants that affected starch metabolism in seeds, as these often led to visible phenotypes on seed morphology. More recently many studies have examined starch metabolism in the model plant *Arabidopsis thaliana* due to the plethora of available insertion mutants, and this has led to a detailed understanding of starch metabolism in photosynthetic tissue. Almost all work examining starch has, therefore occurred in vascular plants. One notable exception to this has been studies examining the unicellular green alga *Chlamydomonas reinhardtii,* where a number of mutants affecting starch metabolism have been isolated (Ball et al., 1996; Buleon et al., 1998; Ball and Morell 2003; Zeeman et al., 2007; Streb and Zeeman, 2012; Busi et al., 2014; 2015; Tuncay et al., 2017' Findinier et al. 2019). Work on such an alga allows a comparison of the pathway of starch metabolism between it, and that established in vascular plants. If it can be shown that the enzymes involved in both vascular and

non-vascular plants influence starch metabolism in the same way, it implies that starch must be advantageous for plant fitness otherwise it would not have been conserved over evolutionary time.

This dissertation describes work examining starch metabolism in the bryophyte *Physcomitrella patens*. In the rest of this chapter, I will describe what starch is and its importance in more detail as well as outline current knowledge about the pathway of its metabolism. I will then describe why I chose to study this in *Physcomitrella* before outlining the aims and objectives of the study.

1.2 The starch polymer

Starch is composed of semi-crystalline granules, which are often round or ovoid in shape and which vary considerably in size from 1 to 100 µm depending on the species (Jobling, 2004). Table 1.1 shows a brief survey of starch characteristics from different plant species. From this it can be seen that starch granules extracted from different plants have a variety of sizes and shapes. Leaf starch granules are generally smaller than granules from storage organs, most likely due to their differing functions in these different organs. Storage organ starches contain small fractions of proteins and lipids which are usually found on the starch granule surface and contribute to the starch's quality and functionality (Alcázar-Alay and Meireles, 2015). For example, some proteins associated with the starch granule of wheat endosperm have received considerable attention as they are associated with grain hardness (Shewry and Halford, 2002; Alcázar-Alay and Meireles, 2015). Starches extracted from different plants also contain small amounts of covalently bound phosphate at the C-3 and C-6 positions of glucosyl units within amylopectin (Blennow et al., 2002). The extent of phosphorylation varies considerably between species and tissue with, for example, cassava root starch containing the relatively low amounts (0.01% (w/w)) and potato tuber starch contains high (0.08% (w/w)) amounts.

Table 1.1 Characteristics of native starch granules from different plant sources (Adapted from Jobling, 2004, Srikaeo and Singchai, 2016; Alcázar Alay and Meireles, 2015).

Species	Granule shape	Diameter (µm)	Lipid (% (w/w))	Protein (%(w/w))	Phosphate (% (w/w))
Arabidopsis leaves	Lenticular, viscoid	1-2	-	-	0.05
Tobacco leaves	Round, spherical	2-10	-	0.02	0.03
Pepper leaves	Round	0.2-7	-	-	-
Potato -Leaves - Tuber	 Oval or round Oval, spherical,	 5 5-100	 - 0.05	 - 0.06	 - 0.08
Wheat	Round, spherical , lenticular	1-45	0.8	0.4	0.06
Cassava	Oval, truncated	2-35	0.1	0.01	0.01
Maize	Round, polygonal	2-30	0.7	0.35	0.02

1.2.1 Amylose and Amylopectin

Amylose and amylopectin are polyglucans present within starch granules. Amylose is regarded as being essentially linear, where the glucose units are linked through α-1,4-glycosidic bonds; however it is known to contain a small number of α1,6 links (Geigenberger, 2011; Streb and Zeeman, 2012; Pfister and Zeeman, 2016). Amylopectin, which makes up the majority of the starch granule (60 to 90% depending on the species), contains many short polyglucan chains where the monomers are joined together mainly by (α-1,4) linkages, but with an additional 5-6% α-1,6-glycosidic branch points (Buléon et al., 1998; Takeda et al., 1984).

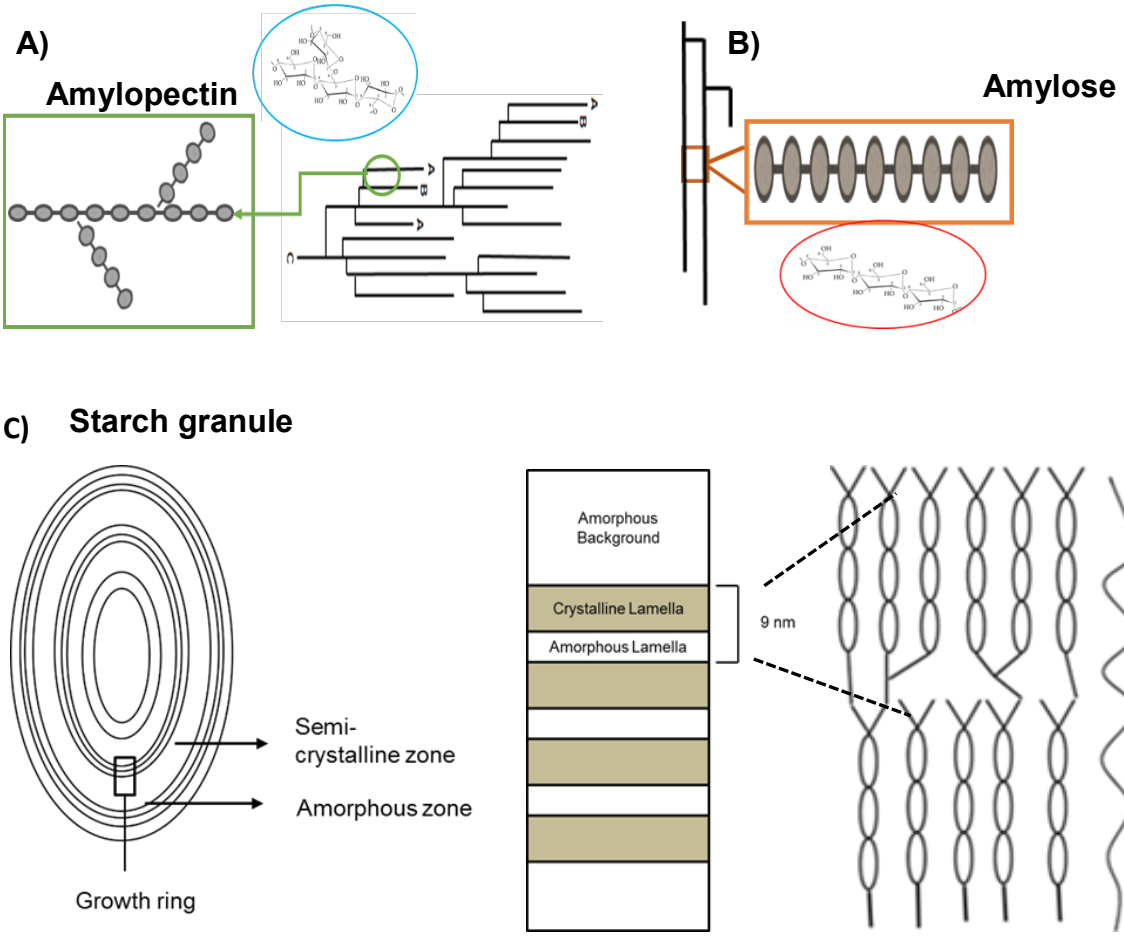

Figure 1.1 Schematic representations of starch granule and the starch polymers. (A) The amylopectin molecule is made up of glucose units linked into chains via α-1,4-glycosidic bonds and branched via α-1,6-glycodic bonds. The molecule consists of (A) chains on the outside and (B) chains on the inside. Both chains are products of the α1-6 branch points. The single (C) chain contains a reducing end. **(B)** Amylose consists of linear chains, linked together by α-1,4-glycosidic bonds. **(C)** The starch granule contains repeated amorphous and semi-crystalline zones, with pairs of these zones making up a growth ring. The semi-crystalline zone is made up of alternating amorphous and crystalline lamellae which occur with a periodicity of 9nm. Crystalline lamellae consist of clusters of amylopectin chains while amylose occurs at the amorphous region (adapted from Gallant et. al., 1997; Buléon et al., 1998; Jobling, 2004; Zeeman et al. 2010).

Internal chains in amylopectin are divided into three categories; **A**, **B** and **C** (Fig. 1.1; Hizukuri et al., 1970; Buléon et al., 1998). **A** chains are found in clusters on the outside of the molecule and

are glycosidically linked to the inner (**B**) chains through the first and sixth carbon of a glucose moiety. The **B** chains in turn can carry **A** and other **B** chains. There is only one **C** chain per amylopectin molecule, which contains the sole terminal glucose moiety and carries all the **A** and **B** chains (Hizukuri et al., 1986; Buléon et al., 1998). Within amylopectin, α1,4 linked chains of about 10 to 20 glucose units are linked together at branch points resulting in the formation of an ordered cluster structure, where clusters of chains occur along the molecule's axis. These are thought to be the **A** and **B1** chains, where **B1** indicates **B** chains that partake in the formation of a single cluster. Amylopectin also consists of longer **B** chains which are responsible for connecting different clusters. These are oriented in the same orientation as the **A** and the **B1** chains and are generally referred to as **B2**, **B3**, and **B4** chains, which span two, three or four clusters of chains respectively (French, 1972; Nikuni, 1978). However, according to Bertoft (2004), it is also possible that these chains are oriented perpendicular to the clusters. It has been revealed through electron microscopy and X-ray scattering that the chain clusters occur at regular intervals of 9 to 10 nm along the axis of the molecule (Buléon et al., 1998; Waigh et al., 1999; Jobling, 2004; Zeeman et al., 2010). This organized arrangement contributes to the crystallinity of the semi-crystalline starch granule (Hizukuri, 1985, 1986; Hanashiro et al., 1996; Buléon et al., 1998; Jobling, 2004; Zeeman et al., 2010).

Amylopectin molecules within the starch granule are radially displayed so that the non-reducing ends of the chains point toward the edge (Zeeman et al., 2010). Within clusters, pairs of adjacent chains form double helices which pack together in well-organized arrays called crystalline lamellae (Waigh et al., 1998). These alternate with amorphous lamellae, which contain most of the branch points. Pairs of repeated stacks of amorphous and crystalline lamellae make up growth rings (Figure 1.1), which can be observed microscopically following digestion of the starch granule with acid or α-amylase (Hoseney, 1994; Waigh et al., 1998). The second glucose polymer within starch, amylose, occupies less space within the granule, something attributed to its linear structure and a low molecular weight compared to amylopectin (Zeeman et al., 2010; Lloyd and Kötting, 2016). Amylose most-likely exists in a disordered form within amorphous lamellae. It has been shown that amylose is not required for starch granule formation as mutant plants lacking

the ability to synthesise it are still capable of producing normal starch granules (Tsai, 1974; Shure et al., 1993).

The production of such an ordered molecule will need the coordinated activities of many enzymes. Over the years, improvements in the field of genomic and transcriptomic analysis has increased our understanding of the biology surrounding starch metabolism and its associated genes and enzymes. The next section will describe the roles of enzymes involved in synthesis and degradation of the starch polymer.

1.3 The biochemistry of starch metabolism

The pathway of starch biosynthesis has been defined through studies on various plants (Deschamps et al., 2008; Nougue et al.; 2014; Pfister and Zeeman, 2016). It is catalysed by several classes of enzymes, with multiple isoforms being present in each class. I will first briefly outline the current model of starch biosynthesis and then examine the enzymes involved in each step in more detail.

The first step of starch synthesis is catalysed by ADP-glucose pyrophosphorylase (AGPase) which uses glucose 1-phosphate (gluc-1-P) and ATP to synthesise ADP-glucose (ADPGlc), the glucosyl donor used for polymer synthesis. Starch synthase (SS) isoforms use the ADPGlc for chain elongation to produce linear polyglucan chains which are branched by starch branching enzymes (SBE) via glucanotransferase reactions.

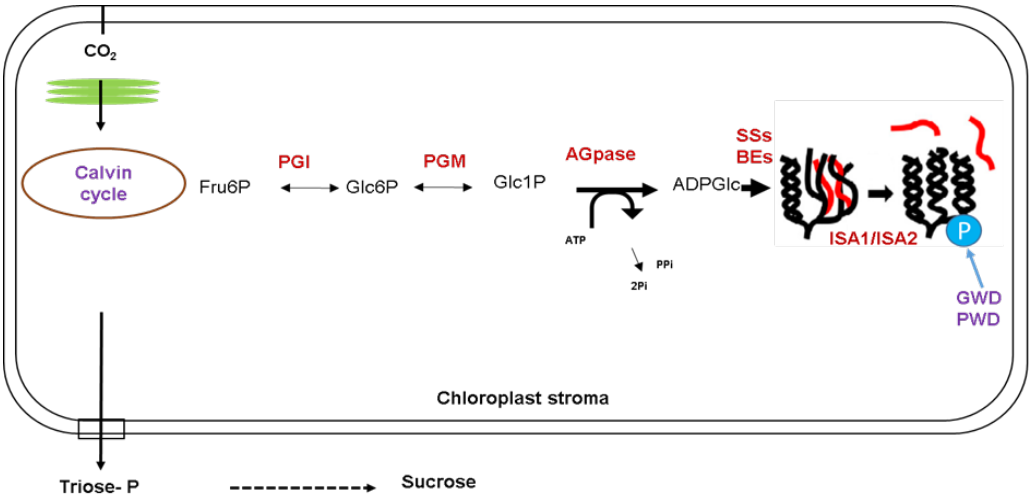

Figure 1.2 An illustration of the starch biosynthesis pathway in leaves. Carbon assimilated via the Calvin cycle is utilized in the chloroplast for starch biosynthesis. SSs and SBEs utilize ADP-glucose to produce phytoglycogen which is hydrolysed by the ISA1/ISA2 complex to form a starch granule.

These three enzyme classes catalyse activities that are sufficient to synthesise both amylose and amylopectin. Over the past 20 years however, it has become apparent that other enzyme classes are also involved. This comes from studies on mutants lacking some isoforms of the isoamylase (ISA) class of debranching enzymes (specifically ISA1 and/or ISA2) which accumulate a highly branched water soluble polysaccharide, phytoglycogen, alongside starch (James et al., 1995; Mouille et al., 1996; Dauvillée et al., 2001; Delatte et al., 2005; Wattebled et al., 2005). Current models of starch metabolism indicate that SS and SBE isoforms synthesise this highly branched molecule and ISA removes excess branch points leading to the formation of amylopectin (Ball et al., 1996; Zeeman et al.,1998).

Although the models proposed to explain the role of ISA isoforms in starch metabolism have provided a good working theory to test, it has become clear that they offer only a partial explanation of starch formation. This comes from observations that plants mutated in either *Isa1* and/or *Isa2* accumulate both phytoglycogen and starch. Indeed, in *Arabidopsis* double *isa1/isa2* mutants some cell types accumulate only starch (Delatte et al., 2005). Both these data

demonstrating that lack of ISA does not lead to elimination of starch. In addition, it has recently become clear that an α-amylase plays a role as, when one α-amylase isoform was mutated alongside all debranching enzymes the plants accumulated increased amounts of starch in addition to phytoglycogen (Streb et al. 2008).

The enzymatic reaction outlined above describes how the starch polymer is formed. The amylopectin fraction often contains some covalently bound phosphate, which is incorporated by two enzymes the glucan water dikinases (GWD) and phosphoglucan water dikinases (PWD; Ball and Morell, 2003; Dauvillee et al., 2006; Jeon et al., 2010; Brust et al., 2013; Liu et al., 2015).

It is clear from this that the way that starch is synthesised is complex and involves many enzyme classes. In the next sections I will examine these in more detail.

1.3.1 ADP glucose - substrate for biosynthesis of the starch polymer

In vascular plants, the substrate for the synthesis of the starch polymer is ADPGlc. In photosynthetic organs, the generation of ADPGlc is directly linked to the Calvin cycle (Figure 1.3). Firstly, fructose-6-phosphate (Fru-6-P) is converted to glucose 6-phosphate (Gluc-6-P) by phosphoglucoisomerase (PGI). Gluc-1-P is then formed from Gluc-6-P by phosphoglucomutase (PGM) and, finally ADPGlc pyrophosphorylase (AGPase) catalyses the conversion of Gluc-1-P and ATP to ADPGlc and pyrophosphate (PP_i; Ghosh and Preiss, 1966; Martin and Smith, 1995; Smith et al., 1997; Ballicora et al., 2003; 2004). Approximately 30-50 % of photoassimilate generated in *Arabidopsis* leaves are partitioned into starch through this pathway (Stitt and Zeeman, 2012).

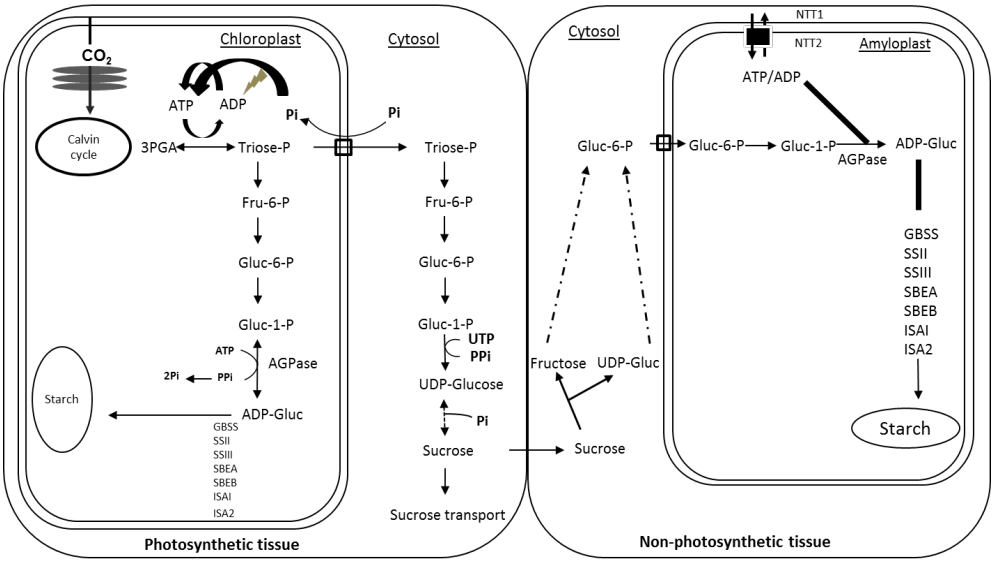

Figure 1.3 Proposed pathway of starch biosynthesis in leaves and storage organs. The carbon fixed in the form of 3PGA is converted to triose-phosphate, which is used for the production of transient starch in chloroplasts. Triose-phosphate can also be exported to the cytosol in exchange with inorganic phosphate (Pi) and then converted to sucrose by a series of enzymatic reactions that liberate Pi. Sucrose is then transported to non-photosynthetic cells for the production of starch (modified from Baroja-Fernández et al., 2004; Ferreira et al., 2010).

Arabidopsis mutants with reduced or abolished activities of PGI, PGM and AGPase have significantly reduced leaf starch contents (Caspar et al., 1985; Lin et al., 1988; Yu et al., 2000). Each of those steps is readily reversible, but PP_i becomes further metabolized by an alkaline pyrophosphatase to orthophosphate (P_i; Gross and Rees, 1986; Weiner et al., 1987; George et al; 2010) which results in the synthesis of ADPGlc being effectively irreversible *in vivo*.

In non-photosynthetic organs, sucrose is imported from leaves via the vascular bundles and used to synthesise ADPGlc (Figure 1.3). Sucrose is converted to Gluc-6-P either by sucrose synthase and UDP-glucose pyrophosphorylase, or a combination of invertase and hexokinase. Glu-6-P is then transported into the amyloplast by the plastidial Gluc-6-P/phosphate translocator (GPT;

Flügge 1999). The imported Gluc-6-P in the amyloplast is converted by plastidial phosphoglucomutase to Gluc-1-P which is used as substrate for ADPGlc synthesis by AGPase (Martin and Smith, 1995; Kammerer et al., 1998; Flügge et al., 2011; Fettke et al., 2011).

The situation is, however, slightly different in endosperm tissue of members of the Poacea family, as these cells possess a cytosolic AGPase that can synthesise ADPGlc in addition to a plastidial AGPase. The ADPGlc synthesised in the cytosol is transported into the plastid by the BRITTLE 1 transporter, in exchange for ADP (Shannon et al., 1998). Endosperm mutants that either lack the cytosolic AGPase or the ADPGlc transporter contain strongly reduced levels of endosperm starch, suggesting the importance of the cytosolic pathway in its synthesis (Sullivan , 1995; James et al., 2003; Johnson et al., 2003; Rosti et al., 2006; Lee et al., 2007; Faix et al., 2012).

The synthesis of ADPGlc by AGPase is regarded as the 'committed' step of starch biosynthesis and is regulated both at the transcriptional and post-translational levels. In vascular plants, AGPase appears as a heterotetramer, consisting of two large (51 kDa) subunits and two small (50 kDa) subunits (Morrell et al., 1987; Okita et al., 1990; Ballicora et al., 2004). The *Arabidopsis* genome encodes four large subunits (APL1- APL4) and two small subunits (APS1 - APS2). These protein subunits evolved from a common ancestor and thus are closely related in sequence but play different roles in determining the kinetic and regulatory properties of the enzyme.

Although generally considered to be regulatory, APL1 and APL2 have been demonstrated to be catalytically active in the presence of a mutated non-catalytic small subunit (APS1), whereas APL3 and APL4 are inactive (Ventriglia et al., 2008). Furthermore, APL1 has been shown to be the main large-subunit isoform in leaves, while APL3 and APL4 are mostly expressed in heterotrophic tissues (Fritzious et al, 2001; Crevillén et al, 2003; 2005; Ventriglia et al., 2008). APS1 is crucial for starch biosynthesis and is catalytically active, whereas APS2 is inactive and lacks an aspartate residue (Asp14) essential for catalysis (Frueauf et al., 2001; 2003; Crevillén et al., 2003).

Regulation of AGPase occurs on several levels. It has been known for several decades that it is allosteriacally regulated by inorganic phosphate which represses its activity and 3-phosphoglyceric acid (3PGA) which activates it (Tuncel and Okita 2013). It is also subject to post-translational redox modulation. Studies on heterologously expressed potato protein in *Escherichia coli* demonstrated that the heterotetrameric protein contains an inter-molecular disulphide bond, linking the two small subunits via cysteine residues. When the disulphide bond was broken, following reduction by dithiothreitol or thioredoxin, the enzyme became activated and the affinity for its substrates increased (Balicora et al.,1998, 1999, 2000; Fu et al., 1998). In *Arabidopsis* leaves, redox activation of the enzyme takes place during the day. It is assumed to be facilitated by thioredoxins and driven by reducing power derived from photosystem I (Buchanan et al., 1980, 2005; Scheibe, 1991).

Such activation of AGPase is also influenced by sugars independently of light *in vivo*. A study in potato tuber by Tiessen et al. (2002), showed redox activation of the enzyme and an increase in the rate of starch biosynthesis in response to high levels of sucrose and glucose. This activation also increases in response to sugar accumulation in leaves of *Arabidopsis*, pea and potato (Hendriks et al., 2003; Gibon et al., 2004; Lunn et al., 2006). In heterotrophic tissues, this redox-activation may be mediated by a plastid-localized NADPH- dependent thioredoxin reductase C (Michalska et al., 2009). Kolbe et al. (2005) demonstrated that trehalose-6-phosphate (Tre-6-P) acts as an intermediary in the sucrose-induced activation of AGPase both in leaves and heterotrophic organs (Kolbe et al., 2005). This was shown by the correlation of the AGPase redox-activation state with the levels of both sucrose and Tre-6-P in seedlings and leaves of *Arabidopsis thaliana* (Lunn et al., 2006). However, more details on the molecular mechanism behind the redox regulation of AGPase and the role of Tre-6-P *in vivo* remain are needed (Hädrich et al., 2012; Streb and Zeeman, 2012). Hädrich et al. (2012) observed that complementation of *Arabidopsis* AGPase-deficient mutant with a redox-insensitive AGPase resulted in faster rates of starch biosynthesis (Hädrich et al., 2012), indicating that this redox activation affects flux through the starch pathway *in vivo*.

AGPase appears to be an important factor that influences the rate of starch biosynthesis in many plants. For instance, starch biosynthesis is severely compromised in AGPase mutants that have lost the predominant isoform of either the small and large subunits in *Arabidopsis* (*adg1* and *adg2*), maize (*brittle2 and shrunken2*) and pea (*rb*) mutants (Tsai and Nelson, 1966; Dickinson and Preiss, 1969; Lin et al., 1988; Smith et al., 1989; Wang et al., 1997; 1998). These mutants contain greatly reduced amounts of starch, providing evidence that AGPase is essential for starch production (Hadrich et al., 2012; Streb et al., 2012). Additionally, Müller-Röber et al. (1992) observed a dramatic loss of starch in potato tubers following the inhibition of AGPase using the antisense RNA approach. Another important factor has been suggested to be the activity of the ATP/ADP nucleotide transporter (NTT), which supplies AGPase with ATP (Neuhaus et al., 1997). Repression of this transporter in potato resulted in decreased amounts of starch in tubers (Tjaden et al., 1998), while its overexpression in potato tubers led to elevated starch levels indicating the importance of the transporter for efficient functioning of AGPase (Zhang et al.,2008).

1.3.2 Starch synthases elongate chains during amylopectin synthesis

Starch synthases (SSs) belong to the glycosyltransferase (GT) family, and catalyse the transfer of glucosyl moeities from ADPGlc to the non-reducing ends of the existing glucan chains (Pfister and Zeeman, 2016). Based on amino acid sequence comparisons, SSs can be divided into at least six classes. Four soluble starch synthases are found in the stroma and are designated, SSI, SSII, SSIII, SSIV and SSV. The final one binds to the starch granule and is named granule bound starch synthase (GBSS; Ral et al., 2004, Patron and Keeling, 2005; Leterrier et al., 2008; Nougue et al., 2014; Pfister and Zeeman, 2016). Although GBSS will be discussed below in more detail in relation to amylose synthesis, I will occasionally compare it to SS isoforms within this section.

Starch synthases contain a catalytic site, that is made up of two glycosyltransferase domains, GT5 and GT1. The GT5 domain includes the acceptor chain binding site, whereas the GT1 domain contains the substrate binding site (Yep et al., 2006; Momma and Fujimoto, 2012; Brust et al., 2013; Cuesta-Seijo et al., 2013). This catalytic domain arrangement is similar to that of glycogen synthases (GSs) in bacteria. Consistent with this many bacteria also use ADP-glucose as a

substrate for glycogen synthesis (Leterrier et al., 2008), indicating the evolution of the starch biosynthesis pathway from that of glycogen biosynthesis (Ball et al., 2011). However, other eukaryotes including yeast, fungi and animals have diverged considerably from plants SSs in sequence. They contain a GT3 domain and use a different sugar, uridine diphosphate glucose (UDPglucose) for the synthesis of glycogen (Deschamps et al., 2008; Zeqiraj et al., 2014; Liu et al., 2015).

GBSS exhibits the lowest molecular weight amongst the SSs, containing GT1 and GT5 domains (Figure 1.4). Although the other SSs also contain these domains, they also demonstrate the presence of additional N-terminal extensions of differing sizes (Leterrier et al., 2008, Pfister and Zeeman, 2016). SSIII and SSIV types have larger N-terminal extensions compared to the other starch synthases. SSIII contains carbohydrate binding molecules (CBMs) at its N-terminus. These have been described as necessary for starch binding (Valdez et al., 2008). SSIII also contains coiled-coil motifs, that are implicated in protein-protein interaction. SSV is a non-canonical starch synthase which contains coiled-coil and GT5 domains (Liu et al. 2015).

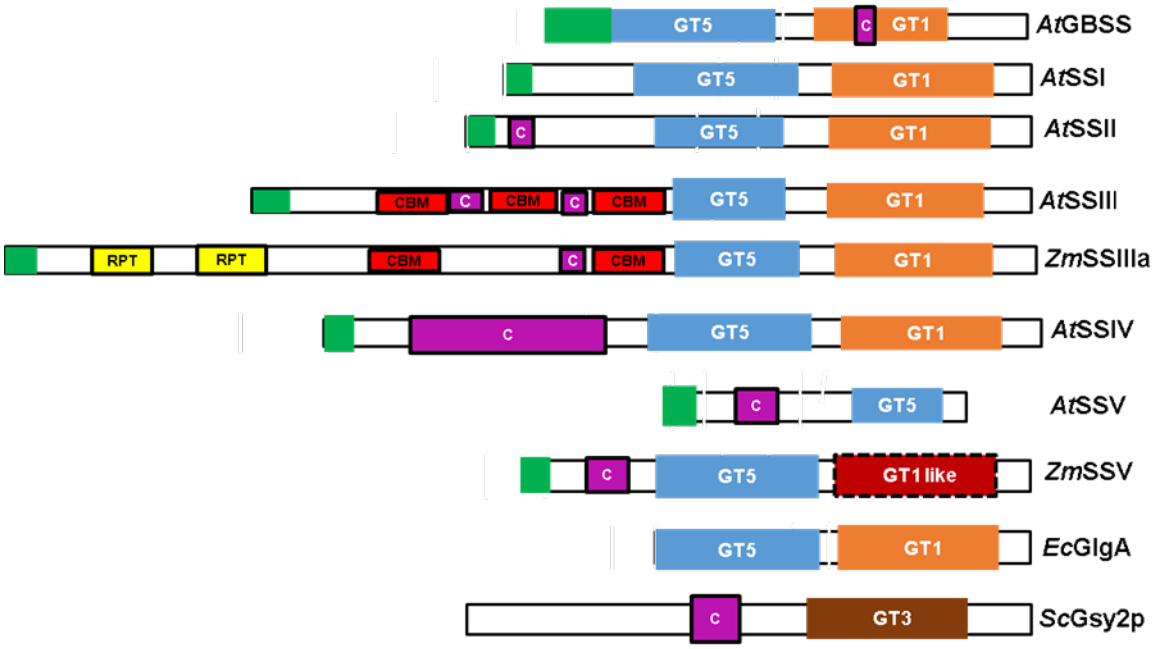

Figure 1.4 Domain structure and comparison of starch synthase (SS) genes. Amino acid sequences from Arabidopsis thaliana (At) were compared with glycogen synthases from Escherichia coli (Ec) and Saccharomyces cerevisiae (Sc). SSIIIa and SSV from Zea mays (Zm) are also included, although they differ in structure compared to the Arabidopsis orthologs. The putative transit peptides (green boxes) were predicted using the ChloroP algorithm. Internal repeats (RPT, yellow boxes), carbohydrate binding molecules (CBM, red boxes), glycosyltransferase-5-domains (GT5, blue boxes), glycosyltransferase-1-domains (GT1, orange boxes), glycosyl transferase-3-domain (grey pattern boxes) and coiled-coil domains (C, pink boxes) are also represented. The coiled-coil motifs were analysed using Paircoil and the rest of the motifs were analysed with SMART.).

SSI, SSII and SSIII are mainly involved in the synthesis of amylopectin and each isoform appears to play a distinct role in its synthesis, although there appears to be some overlap between some of the isoforms. Analyses of mutant plants lacking each of the SS isoforms suggest that they have similar effects in all plant species analysed so far, although the tissue in which the starch was produced may vary, depending on the botanical source. Their roles, therefore, seem to be conserved among the different starch producing species (Seung, 2017). SSI is responsible for the synthesis of short chains, approximately 10 or fewer glucosyl units. Loss of SSI activity in *Arabidopsis* result in a decreased abundance chains of short chains (DP less than 13), suggesting that SSI plays a major role in the production of these short glucans (Delvallé et al., 2005). Rice endosperm *ssI* mutants displayed the same phenotype (Fujita et al., 2006), although no difference could be found in starch from transgenic potato tubers lacking SSI (Kossmann et al. 1999)

SSII is responsible for the synthesis of medium-length chains. The role of SSII have been investigated in a number of plants including potato tubers (Kossmann et al., 1999, Edwards et al., 1995,1999, Lloyd et al., 1999) pea seeds (Craig et al., 1998), wheat endosperm (Yamamori et al., 2000), rice (Umemoto et al., 1999; Umemoto et al., 2002), barley endosperm (Morell et al., 2003) and *Arabidopsis* leaves (Zhang et al., 2008; Pfister et al., 2014; Szydlowski et al., 2011) through the isolation of mutants or production of transgenic plants. These all lead to a decreased abundance of medium length chains (DP between 12-30) within amylopectin, often accompanied by drastic altered granule morphology and elevated amylose contents.

The role played by SSIII is believed to be in the elongation of longer chains (DP >30, **B2** & **B3** cluster chains) and in starch granule initiation (discussed in the context of SSIV below). Elimination of SSIII resulted in starches with fewer of these chains, in potato tubers (Edwards et al. 1999; Lloyd et al., 1999), maize endosperm (Inouchi et al.,1987; Wang et al., 1993), rice endosperm (Fujita et al., 2007) and *Chlamydomonas reinhardtii* (Ral et al., 2006). However, the *ssIII* mutation in *Arabidopsis* had less impact, leading to only small alterations in the structure of amylopectin (Zhang et al., 2008). Interestingly, there appears to be some functional overlap

between SSII and SSIII. Evidence to this was supported by the drastic decrease in the abundance of the medium-length chains, observed as a result of the simultaneous mutation or antisense repression of SSII and SSIII. This reduction was even greater than that observed in plants repressed in *ssii* alone (Edwards et al. 1999; Lloyd et al. 1999; Zhang et al., 2008).

Unlike the other starch synthases, SSIV and SSV seems to have a little influence on the structure of amylopectin and amylose (Roldán et al., 2007; Szydlowski et al., 2009; Abt et al. 2020), however, they are believed to be playing a major role in starch granule initiation. *Arabidopsis ssiv* and *ssv* mutants demonstrate remarkable alterations in the shape and number of starch granules. Instead of six discoid granules, the mutant appears to contain zero to two granules per chloroplast. These are altered in shape, being enlarged, spherical and less electron-dense (Roldán et al., 2007; Crumpton-Taylor et al., 2013; Malinova et al., 2017; Abt et al. 2020). Interestinglythe *ssiv* mutant has elevated ADPGlc levels, suggesting that consumption of this metabolite is limited, meaning that the other SSs are unable to use it in the absence of SSIV (Crumpton-Taylor et al., 2013; Ragel et al., 2013). On the other hand, *Arabidopsis* ssI/ssII/ssIII triple mutants which have SSIV and SSV as the sole soluble SS's, yield similar numbers of granules in their chloroplast as the wild type, even though they contain, generally, little aberrant starch (Szydlowski et al., 2009). Additionally, there appears to be a developmental switch in starch accumulation as young leaves from *ssiv* plants were observed to have starch-free chloroplasts (Crumpton-Taylor et al., 2013). Overexpression of *AtSSIV* in *Arabidopsis* led to an increase in total leaf starch content, even though there was no increase in the number of starch granules observed (Gámez-Arjona et al., 2011). Taken together, these results show that *Arabidopsis* SSIV and SSV initiate starch granule formation (Roldán et al., 2007; Crumpton-Taylor et al., 2013; Abt et al. 2020). An *Arabidopsis ssiii/ssiv* double mutant is essentially starchless, indicating that SSIII acts alongside SSIV and SSV in initiating starch granule initiation (Szydlowski et al., 2009).

1.3.3 What facilitates the branching of the glucan chain?

The formation of amylopectin would be incomplete without the contribution of the branching enzymes (BEs). Their roles are crucial, as the distribution of branch points within amylopectin helping to determine the cluster structure and physical properties of starch. They act by cleaving internal α1,4 linkages from donor chains and then transfer them to acceptor chains via the α-1,6-glycosidic linkages, resulting in a branch point (Borovsky et al., 1975; Pfister and Zeeman,2016). Analyses of primary sequences show that BEs are composed of three domains (Figure 1.5). This includes an N-terminal domain (containing the CBM of family 48), a central catalytic α-amylase domain (characteristic for GH13 family members) and a C-terminal domain (present in most α-amylases).

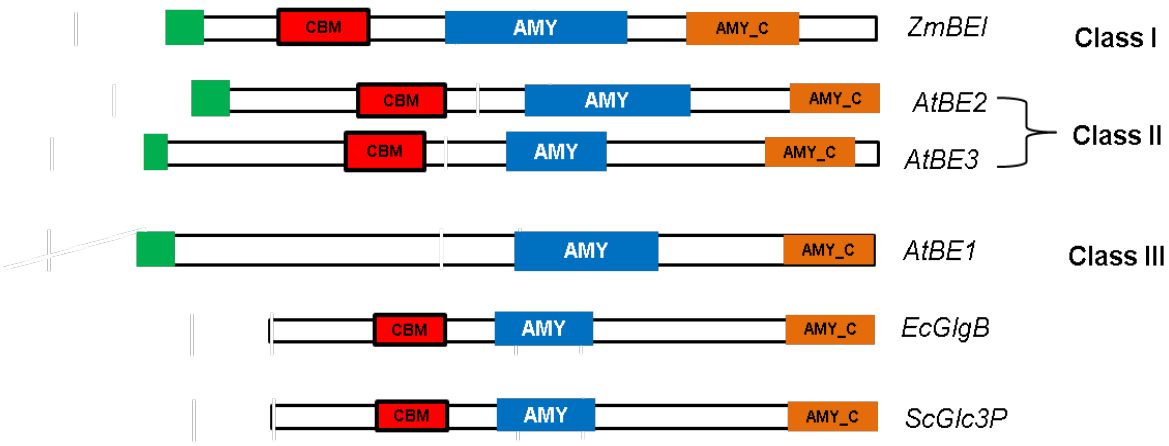

Figure 1.5 An illustration of the domain structure of SBE enzymes. Represented are the N-terminal transit peptide (green boxes), the CBM of family 48 (red boxes), the catalytic α-amylase family domains (AMY, orange boxes) and the β-domains (AMY_C, blue boxes), found in the C-terminus of α-amylases. Domain structure appears to be conserved between orthologs, but the length of the catalytic α-amylase family domain varies. BEI from *Zea mays* (*Zm*BEI) is included for comparison since Arabidopsis lacks the class I BE. Glycogen BE's from *E. coli* (*Ec*GlgB) and *Saccharomyces cerevisiae* (*Sc*Glc3p) are also included. The putative class III BE (*At*BE1), which does not contain the CBM48, is also shown (structure adapted from Pfister and Zeeman, 2016 with modification).

In vascular plants, SBEs are divided into two classes, class I or family B (BEI) and class II or family A (BEII; Burton et al., 1995; Pfister and Zeeman, 2016). BEI have a high affinity for amylose and preferentially transfer longer chains than BEII. In contrast, the BEII subclass preferentially branch amylopectin (Pfister and Zeeman, 2016). Mutants with reduced activity of BEI have been analysed in various plants. These include maize (Blauth et al., 2002; Xia et al., 2011), rice (Satoh et al., 2003; Abe et al., 2014), and wheat (Regina et al., 2004) endosperms as well as tubers from transgenic potato tubers (Safford et al., 1998). The elimination or reduction of BEI in all cases, resulted in only small changes on the structure and amount of starch accumulated (Safford et al., 1998; Blauth et al., 2002; Satoh et al., 2003). Mutations in BE1 type isoforms, however, do result in an increase in amylose fraction within starch. Examples of this include the *rugosus* mutant of pea (Bhattacharyya et al. 1990) alongside barley transgenic and mutants (Regina et al. 2010; Carciofi et al. 2012), and and potato mutants introduced using CRISPR/cas 9 (Zhao et al 2021). This indicates a major role for BE1 type isoforms in the synthesis of branchpoints within amylopectin.

In *Arabidopsis*, there are two BE isoforms, BE2 and BE3. Both isoforms are expressed in leaves and appear to be functionally redundant (Dumez et al., 2006). Mutations in either BE2 or BE3 do not affect the total starch content and result also in similar minor alterations in the amylopectin structure. However double mutation between these isoforms leads to accumulation of large amounts of maltose instead of starch, suggesting that without the branching activity, all linear glucans produced are broken down (Dumez et al., 2006).

1.3.4 Debranching enzymes determine the final structure of amylopectin

In addition to the actions of the starch synthases and starch branching enzymes, a third class of enzyme (debranching enzymes; DBE) is required for the synthesis of starch with a normal structure (Zeeman et al., 2010). DBEs catalyse the hydrolysis of α-1,6-linkages in branched glucan polymers and determine a clustered arrangement of branched linkages in precursor glucans (Nakamura et al., 1997; Myers et al., 2000; Streb et al., 2008; Streb and Zeeman., 2012). They form part of the glycoside hydrolase families (GH13 and GH57) and share the central catalytic α-

amylase and starch binding domains with BEs (Figure 1.5,1.6; http://www.cazy.org/; Lombard et al., 2014).

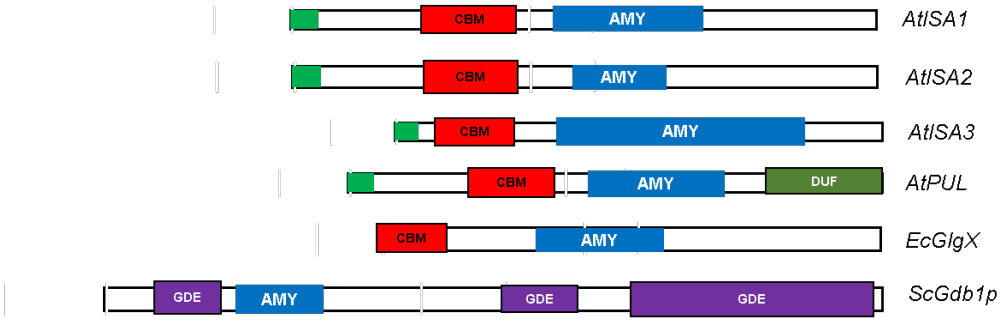

Figure 1.6 An illustration of the domain structure of DB enzymes. The Arabidopsis DBEs (*At*ISA1, *At*ISA2, *At*ISA3 and *AtPUL*) and *E. coli* DBE (*Ec*GlgX) have a similar structure with that of the BEs. Represented are the plastidial transit peptides (green boxes), the CBM of family 48 (red boxes), the catalytic α-amylase family domains (AMY, blue boxes) and domains of unknown functions (DUF, dark green box). The *S. cerevisiae* DBE (*Sc*Gdb1p) is included for comparison and has three glycogen-debranching enzyme (GDE) domains.

Plant DBEs are divided into two classes, namely isoamylase (ISA) and limit dextrinase (LDA). These are distinguishable by their protein sequences and substrate specificities. For instance, LDA prefers pullulan to glycogen or amylopectin and, for this reason, it is sometimes referred to as pullulanase. Pullulan is a yeast derived glucan, which consists of α-1,6-linked maltotriosyl units. The isoamylase class can be further subdivided into three isoforms: ISA1, ISA2 and ISA3 (Hussain et al., 2003; Rahman et al., 2003). ISA1 and ISA2 are primarily associated with amylopectin synthesis. In contrast, ISA3 and PUL are mainly involved in starch degradation (Wattebled et al., 2005; Delatte et al., 2006; Streb et al., 2012).

There is strong evidence that suggests that ISA1 is an active enzyme, while ISA2 is catalytically inactive, most probably due to changes in key amino acids within the active site (Macgregor, 1993; Hussain et al., 2003; Sundberg et al., 2013). ISA1 and ISA2 usually associate as a

heteromultimeric enzyme complex (Bustos et al., 2004; Sundberg et al., 2013). The existence of this complex has been confirmed in *Chlamydomonas* (Dauvillé et al., 2001), potato tubers (Bustos et al., 2004) and *Arabidopsis* leaves (Delatte et al., 2005). In this complex, ISA2 is suggested to have a regulatory function and is also proposed to confer substrate specificity (Katsuya et al., 1998; Hussain et al., 2003; Deschamps et al., 2008; Woo et al., 2008; Sim et al., 2014). The complex is unstable when either protein partner is eliminated, so inhibition of either of the genes encoding ISA1 or ISA2 will affect its formation. In some plants ISA1 alone has been shown to form an active homomultimer complex alongside the ISA1/ISA2 heterocomplex. At least one ISA1 homomeric complexes has been found in the endosperm of rice (Utsumi and Nakamura, 2006), maize (Kubo et al., 2010) and in *Chlamydomonas reinhardtii* (Dauville et al., 2001; Sim et al. 2014). Rice and maize *isa2* mutants, with contain only the ISA1 homomeric complex accumulate endosperm starch to the same amount as wild type, suggesting that the ISAI homomer is more important that the heterocomplex in these tissues (Utsumi et al., 2011; Kubo et al., 2010).

It is believed that ISA1 and ISA2 remove branch points that have been wrongly positioned by BEs which are thought to hinder the biosynthesis of the semi-crystalline amylopectin fraction of starch (Zeeman et al., 2010). Transgenic and mutant plants with reduced amounts of ISA1 accumulate reduced amounts of starch and increased amounts of a highly branched, water soluble polysaccharide named phytoglycogen. Its accumulation in *isa1* mutants has been documented in a variety of plants including barley (Burton et al., 2002), rice (Nakamura et al., 1997), maize (James et al., 1995), *Chlamydomonas reinhardtii* (Dauville et al., 2001; Mouille et al., 1996; Posewitz et al., 2004), *Arabidopsis* (Wattebled et al., 2005; Delatte et al., 2005) and potato (Bustos et al., 2004).

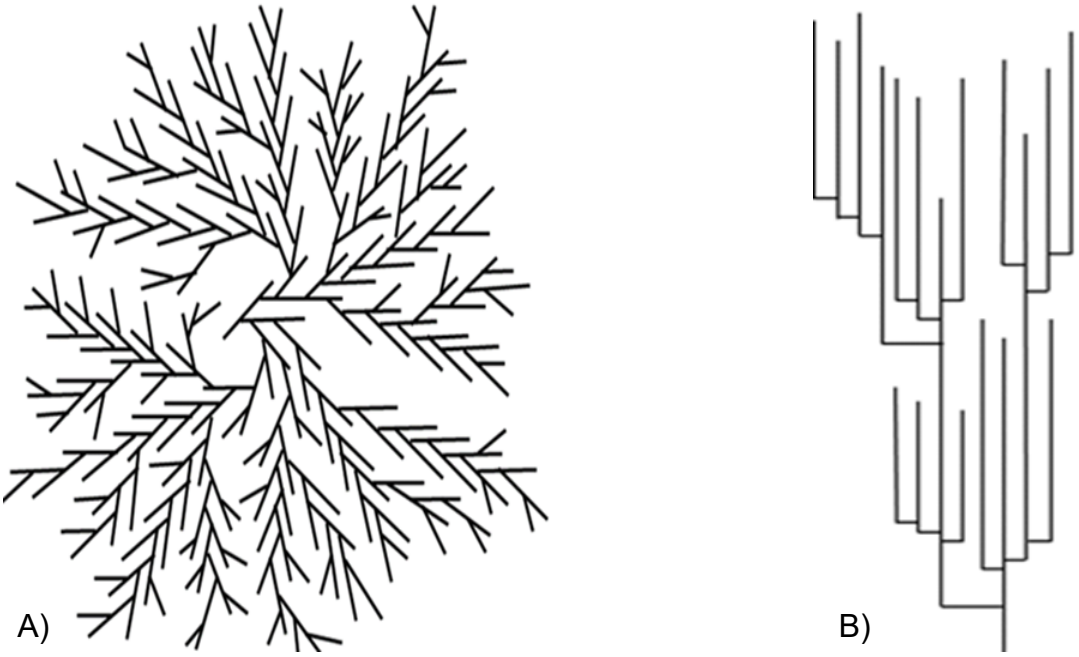

Figure 1.7 Representation of (**A**) the branching pattern found in phytoglycogen and (**B**) regular cluster-like structure of amylopectin (Adapted from Nakamura, 2002)

Phytoglycogen resembles glycogen in structure (hence its name), with shorter average chain lengths and a higher degree of branching compared with amylopectin (Figure 1.7; Summer and Somers, 1944).It is believed that the branching structure hinders the production of the higher order structures observed in normal starch granules (Zeeman et al., 2010). This means that the extra branchpoints need to be removed through debranching activity for starch to form (Ball et al., 1996; Zeeman et al., 2007; Streb et al., 2008). The liberated chains are released as free oligosaccharides, leaving a mature amylopectin molecule. If the wrongly-positioned branches are not removed, the glucan cannot undergo a process of self-organization to form a granule leading to the accumulation of phytoglycogen (Ball et al., 1996; Myers et al., 2000; Nakamura, 2002; Zeeman et al., 2007).

Mutations in ISA 1 lead to phytoglycogen accumulation in a wide range of plants, from green algae to cereals (James et al. 1991; Zeeman et al. 1998; Wattebled et al. 2005, 2008; Sim et al.

2014), however, the consequences of mutations in ISA2 varies depending on plant species. In *Arabidopsis* and potato, elimination of either ISA1 or ISA2 results phytoglycogen accumulation (Bustos et al. 2004; Delatte et al. 2005, Wattebled et al., 2005). This can be explained by the presence of a heteromultimeric ISA1/ISA2 complex in this tissue where loss of either enzyme destabilizes the remaining protein subunit (Sundberg et al., 2013). In contrast, loss of ISA2 in rice and maize endosperms has no obvious effect on starch as these cereal tissues still contain active homomultimeric complexes of ISA1 (Kubo et al., 2010; Utsumi et al 2011).

1.3.5 GBSS plays a major role in amylose biosynthesis

Although amylose is smaller than amylopectin, with only a 100 to 10000 glucose molecules, it has received much attention because of its functional properties that have made it an important commodity in the food and non-food based industries (Jane et al., 1999; Jobling 2004; Van Hung et al., 2006; Santelia and Zeeman, 2010; AAF, 2013). The key enzyme responsible for amylose synthesis is GBSS, which is tightly bound to the starch granule (Shure et al., 1983; Imam, 1989; Flipse et al., 1994; Denyer et al., 2001). Mutants that lack GBSS in the endosperms of maize (Sprague et al., 1946; Shure et al., 1983), rice (Sano, 1984; Wang et al., 1995), wheat (Nakamura et al., 1995), barley (Patron et al., 2002), amaranth (Konishi et al., 1985), cassava roots (Ceballos et al., 2007), potato tuber (Hovenkamp-Hermelink et al., 1987), pea seeds (Denyer et al., 1995), *Arabidopsis* leaves (Seung et al., 2015), and even *Chlamydomonas* reinhardtii (Delrue et al., 1992; Ral et al., 2006) all produce starch with reduced amounts of, or no, amylose. Additionally, the expression of an antisense sequence of the GBSS gene resulted in a drastic reduction of amylose in transgenic potato and rice (Visser et al. 1991; Shimada et al., 1993; Kuipers et al. 1994). There is very little, or no, reduction in starch contents in these mutant and transgenic plants. Mutations in *GBSS* are often called '*waxy* ', and in addition to reduced amylose levels, contain sticky glutinous starch, which is very useful in the food industry (Jobling, 2004).

1.3.6 The pathway of starch degradation

A large proportion of the starch produced during the day is degraded at night, to provide energy to sustain plant growth and metabolism (Smith et al., 2005). Much has been learned about this pathway through the study of *Arabidopsis thaliana* mutants impaired in starch degradation which accumulate starch, and display decreased growth rate and a starch excess phenotype (Caspar et al., 1991). Starch degradation takes place through a series of hydrolytic reactions that result in the liberation of the end-products glucose and maltose, which are in turn transported into the cytosol for the production of sucrose (Fig. 1.8; Heldt et al., 1977; Peavey et al., 1977; Herold et al., 1981; Stitt & Heldt, 1981; Neuhaus & Schulte, 1996; Schleucher et al., 1998; Servaites & Geiger, 2002; Ritte & Raschke, 2003; Chia et al., 2004; Weber, 2004; Lloyd et al., 2005).

The first step of starch degradation appears to be the destabilization of double helical glucan chains at the surface of the insoluble starch granule by the phosphorylating enzymes glucan water dikinase (GWD1) and phosphoglucan water dikinase (GWD3/PWD; Loberth et al., 1998; Yu et al., 2001; Ritte et al., 2002; 2006; Baunsgaard et al., 2005; Kötting et al., 2005; Edner et al., 2007; Hejazi et al., 2009). GWD1 catalyses the phosphorylation of starch at the C6 position of the glucose monomers, using ATP as phosphate donor (Ritte et al., 2002). Vascular plants with reduced GWD1 activity show both a decrease in starch phosphate content and a starch excess (SEX) phenotype in leaves (Caspar et al., 1991; Lorberth et al. 1998; Yu et al., 2001; Nashilevitz et al. 2009; Hirose et al., 2013). PWD on the other hand, phosphorylates starch at the C3 position of the glucosyl units, and has been reported to strongly depend on pre-phosphosphorylation of the starch molecule by GWD1 prior to its action. Mutations in PWD also lead to a starch-excess phenotype, although not as pronounced as with mutations in GWD1 (Baunsgaard et al., 2005; Kötting et al., 2005).

Starch phosphorylation appears to be conserved in the plant kingdom, with GWD isoforms having been reported in many species including unicellular green algae, cereals, potato and *Arabidopsis* amongst others (Lorberth et al., 1998; 1998; Ritte et al., 2000; Blennow, 2015; Findinier et al. 2019). It is believed that the actions of these enzymes disrupt and destabilize the

helical structure of amylopectin and expose it to degradative enzymes. Subsequent degradation of starch through the activity of endo- and exo-amylases, such as α-amylases3 (AMY3) and β-amylase3 (BAM3; Ritte et al., 2006; Zeeman et al., 2007; Pfister and Zeeman, 2016). These enzymes result to the formation of phosphorylated malto-oligosaccharides (MOS), where the phosphate monoesters need be eliminated prior to being degraded further (Takeda and Hizukuri, 1981; Edner et al., 2007). Two phosphatases, Starch Excess 4 (SEX4) and Like SEX4 2 (LSF2) have been characterized in *Arabidopsis*. SEX4 has been shown to be active on phosphate covalently bound both at the C6 and C3 positions, while LSF2 eliminates phosphate groups attached to the C3 position only (Kötting et al., 2009; Santelia et al., 2011). Mutations in genes encoding either of these enzymes, or transgenic plants where their expression was repressed, result in the accumulation of phosphorylated MOS and an inhibition in starch degradation (Kötting et al., 2009; Santelia et al., 2011; Silver et al., 2014; Samodien et al., 2018).

The released dephosphorylated MOS are hydrolysed by BAMs (as mentioned above), but these enzymes can only act on α-1,4 linkages and not on α-1,6-branch points, and therefore require the assistance of the debranching enzyme, particularly, ISA3, for complete hydrolysis of amylopectin (Baba and Kainuma, 1987; Scheidig et al., 2002; Delatte et al., 2006). BAMs produce maltose, but are unable to hydrolyse chains that are shorter than three glucose molecules. As a result, maltose and maltotriose are released into the stroma. A plastidial disproportionating enzyme (DPE1) acts on the maltotriose leading to the formation of glucose and longer glucan chains (malto-pentaose), which are hydrolysed further by β-amylases (Kakefuda and Duke, 1989).

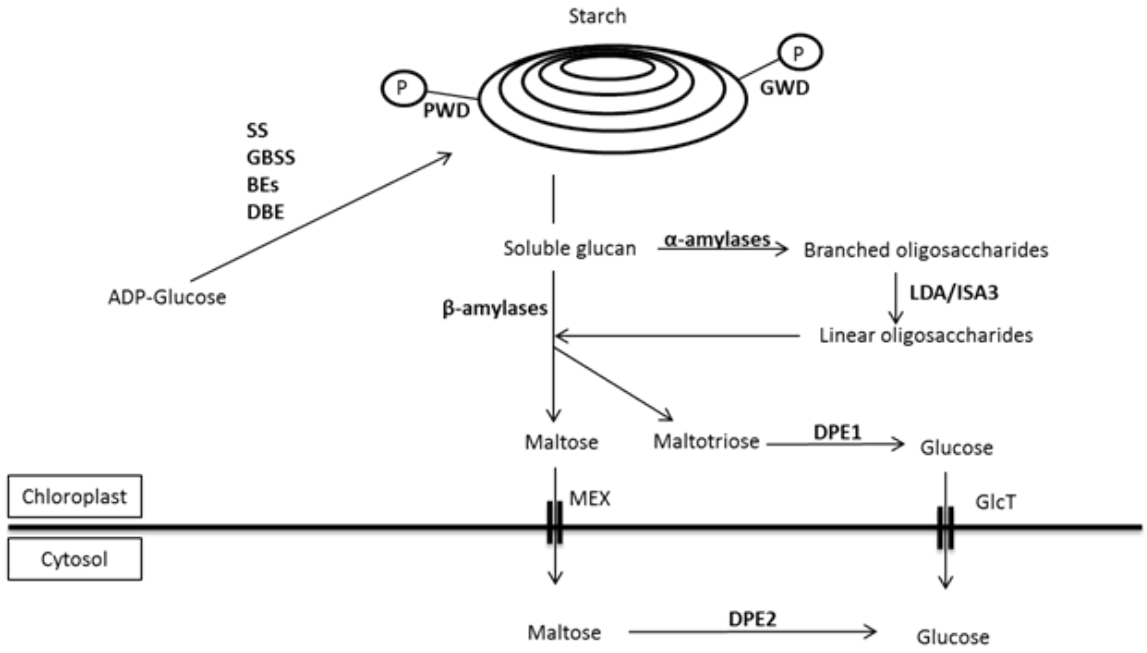

Figure 1.8 Proposed pathway of starch degradation in Arabidopsis. GWD and PWD incorporate phosphate groups to the starch granule, making it accessible to the α-amylases and β-amylases. The activities of these enzymes lead to the production of maltose, glucose and maltotriose. Maltose is exported to the cytosol by MEX where it is further converted to glucose by DPE2. Maltotriose is degraded in the stroma to glucose by DPE1. Glucose is then exported to the cytosol (Adapted from Wiese et al., 2006; Stander, 2015).

Maltose and glucose are transported from the chloroplast into the cytosol by the specific transporters, MEX1 and pGlcT proteins respectively (Schafer et al., 1997; Weber et al., 2000; Servaites and Geiger, 2002; Niittyla et al., 2004; Cho et al., 2011). *Arabidopsis thaliana mex1* mutants accumulate large amounts of starch and maltose, and display a distinctive growth retardation phenotype indicating the importance of this transporter in plant growth (Niittylä et al., 2004; Lu et al., 2006). Recent work on *Chlamydomonas reinhardtii* has revealed that, unlike *Arabidopsis thaliana*, mutations in a *MEX1* like gene does not result in accumulation of maltose. Further investigations revealed that the algal protein is capable of glucose transport, while the

Arabidopsis MEX can transport both maltose and glucose. This indicates a change in the transport specificity of this protein has changed during colonisation of land by plants (Findinier et al., 2017). In *Arabidopsis* maltose is further metabolized in the cytosol by the combined activities of a second disproportionating enzyme (DPE2) and cytosolic phosphorylase (PHS2). These enzymes convert maltose into glucose, which is subsequently used to maintain sucrose metabolism (Chia et al., 2004; Lloyd et al., 2004; Lu and Sharkey, 2004; Smith et al., 2004; Weber et al., 2004). Therefore, from these data, it is clear that starch degradation in *Arabidopsis* occurs via two distinct routes. The first leads to the production of glucose within the plastid, while the other involves the hydrolysis of maltose outside the plastid. The latter route appears to be the main pathway through which most of the flux from the chloroplast flows, as mutants disrupted in this pathway (*mex1 and dpe2*) show a more severe repression of starch degradation (Lu & Sharkey, 2004; Chia et al., 2004; Lloyd et al., 2004; Niittylä et al., 2004) than *pglct-1* and *dpe1* mutants (Critchley et al., 2001; Chia et al., 2004; Niittyla et al., 2004; Lütgen et al. 2010; Cho et al., 2011).

1.3.7 A new class of proteins involved in starch metabolism

Recently, Seung et al. (2016; 2017) have discovered a family of proteins, named Protein Targeting to Starch (PTST). These are glucan-binding proteins with coiled-coil motifs and, in *Arabidopsis*; there are three isoforms (PTST1, PTST2 and PTST3), while rice contains *Os*FLO6 (Peng et al., 2014) orthologous to PTST2. Orthologs have also been identified in green algae and many land plants including barley and cassava (Seung et al., 2015; Bull et al., 2018, Wang et al., 2019). However it appears that some members of the grass family lack the PTST3-like isoform (Seung et al., 2015; 2017). PTST1 plays a critical role in the production of amylose, as it enables GBSS to bind and localise to the starch granule. Further investigation revealed that PTST physically interacts with GBSS via the coiled-coils. Likewise, *Arabidopsis* mutant plants deficient in PTST1 produce an amylose-free starch (Seung et al., 2015) while mutations in other species leads to reduced amylose or elimination of starch accumulation (Bull et al., 2018, Wang et al., 2019, Zhong et al., 2019).

PTST2 interacts with SSIV, which is required for normal granule initiation alongside 2 other proteins known to be involved in starch granule initiation MYOSIN-RESEMBLING CHLOROPLAST PROTEIN and MAR BINDING FILAMENT LIKE PROTEIN1 (Seung et al., 2018, Vandromme et al., 2019). Chloroplasts in *Arabidopsis ptst2* mutants contained one large granule or none. In contrast, overexpression of PTST2 led to the accumulation of many tiny granule and further experiments demonstrated that PTST2's CBM48 domain interacts with long malto-oligosaccharides. No interacting partner for PTST3 has yet been identified, but *ptst3* mutants showed a reduction in the number of granules compared to the wild-type and double mutants lacking PTST2 and PTST3 resulted in fewer granules than those observed in *ptst2*. Taken together, these data indicated the critical role played by PTST2 and PTST3 in starch granule initiation potentially by binding to malto-oligosaccharide primers and then delivering them to SSIV (Seung et al., 2017).

1.4 Evolution of the pathway of starch synthesis

About 1.5 billion years ago, an archaeplastidial eukaryote entered into symbiosis with a photosynthetic bacterium (Ball et al., 2005). This second major endosymbiotic event had far reaching consequences resulting in the evolution of three eukaryotic lineages: the Glaucophyta (unicellular algae), Rhodophyta (red algae) and Chlorophyta (green algae and land plants) which have been classified as Archeaplastida (Adl et al., 2005; Deschamps et al., 2008a).

Prior to endosymbiosis, the host organism synthesised glycogen via UDP-glucose, while the endosymbiont utilized ADP-glucose. When endosymbiosis was complete, both UDP-glucose and ADP-glucose were utilized for starch biosynthesis, a process which required enzymes from both participants (host organism and symbiont). This allowed for the export of ADP-glucose produced by the acquired cyanobiont to the host cell cytosol, where it was polymerized into starch, thereby achieving the transfer of photosynthate and establishing a metabolic link, between the two organisms (Deschamps et al., 2008a; Ball et al., 2011). It is generally believed that when Glaucophyta and Rhodophyta diverged from the Archaeplastida, starch production still remained in the cytoplasm with the use of UDP-glucose as substrate, while it was redirected to the plastid

that had remained the site of ADP-glucose synthesis in Chlorophyta (Deschamps et al., 2008b, 2008c; Ball et al., 2011). The enzymes used for starch elongation in the Chlorophyta thus were dependent on those that originally came with the cyanobiont, as these were already adapted to the utilization of ADP-glucose. While cytosolic starch is found in the cytoplasm of several lower plants species including Glaucophyta, it is specifically known as "floridean" starch when produced within a particular group of red algal species called Florideophycidae (Viola et al., 2001; Ball et al., 2011).

Higher temperatures have been reported to negatively affect floridean starch, displaying lowered viscosity and gelatinization temperatures, but leave plastidial starch unaffected. This offers an evolutionary advantage to survival at higher temperatures (Yu et al., 2002; Deschamps et al., 2008) which may be responsible for the presence of plastidial starch in land plants. *Bryophytes* are counted amongst the early diverging lines of Viridiplantae, colonising land about 450 million years ago. This was accompanied by more extreme temperature conditions alongside increased exposure to ultraviolet radiation and decreased water availability. As a result, adaptations involved changes in structure and organization of the plants cellular, physiological and regulatory processes. These included development of osmoprotectants and osmoregulators, improved freezing and desiccation tolerance, thermal resistance, production of sun protection material and improved DNA repair processes. It is plausible that, these events were also coupled with genomic changes including the expansion and complexity of gene families, loss of genes that improve fitness in an aquatic environment and acquisition of genes helping survival in terrestrial environments. Fossil evidence shows that early land plant species were structurally similar to *Byrophytes* (Lang, 1937; Gray, 1985; 1993; Edwards et al., 1995; Kenrick and Crane 1997; Wellman and Gray 2000; Edwards and Kenrick 2015). Comparison of genomes from early land-colonising non-vascular plants, such as *P. patens*, with Angiosperms offers a way to understand how metabolic pathways have evolved and diverged since land settlement.

Decades of research in starch metabolism have led to the identification and increased understanding of a well conserved pathway, from the earliest diverging unicellular green algae, *Ostreococcus* to multicellular plants (Ral et al., 2004; Derelle et al., 2006; Deschamps et al., 2008).

This pathway seems to solely rely on ADP-glucose for the production of starch (Ral et al., 2004). The enzymes responsible for the production of ADP-glucose and those that elongate glucosyl units within this substrate show a distinctive bacterial phylogeny, which correlates with the plastidial location of starch in Chlorophyta (Coppin et al., 2005; Patron and Keeling , 2005; Deschamp et al., 2008). Research has shown that gene duplications have contributed enormously to the organization and diversification of the starch biosynthesis pathway in this kingdom, and detailed sequence analysis in *Arabidopsis* revealed that, approximately 18% of the genes can be traced back to the endosymbiont (Martin et al., 2002). It is believed that the majority of the starch biosynthesis genes emerged from these duplications, during the course of the Archaeplastidial evolution (Zhang, 2003). The soluble starch synthases SS III-IV and the isoamylases derived from the lateral gene transfer from the species of pathogenic bacteria, *Chlamydia.* The pullulanases, on the other hand, were acquired from unidentified probacterial sources (Deschamp et al., 2008; Ball et al., 2011). However, some gene duplications have only occurred recently, specifically that of GBSS especially within the grass family (Nougué et al., 2014).

1.5 Physcomitrella patens as an organism to study starch metabolism

The bryophyte *Physcomitrella patens (P. patens)* is a member of one of the oldest groups of terrestrial plants which had diverged from seed plants more than 400 million years ago (Cove, 2005). It is widely distributed, and isolates are available from North America, Europe, Africa, Australia and Asia (Cove, 2005; McDaniel et al., 2010). *P. patens* typically grows in dense clumps or mats, on banks of ponds, rivers, lakes or shady areas (Cove, 2005). It still retains many of the structures and features of vascular plants, and often responds to environmental changes and phytohormones in a similar manner to that of vascular plants (Hirano et al., 2007). It has been reported that at least about 66% of *Arabidopsis thaliana* genes share strong sequence similarities with those found in *P. patens* (Nishiyama et al., 2003).

The genome of the Gransdan strain of *P. patens* is approximately 500 Mbp and has been sequenced a (Rensing et al., 2007; 2008). The assembled genomic sequence is estimated to contain about 36000 predicted and annotated genes.

1.5.1 *Physcomitrella patens* life cycle

The life cycle of *P. patens* (Fig. 1.9) is composed of alternation between haploid (entails gamete production) and diploid phases (generation of haploid spores via mitosis; Cove, 2005). A haploid spore germinates and produce protonemal tissue, which consists of two types of filamentous cells. Chloronemal filaments consist of densely packed cells containing large and well-developed chloroplasts while caulonemal tissue develops from chloronema and contains longer cells with fewer and less developed chloroplasts (Reski, 1998; Cove, 2005). Buds may eventually develop from the caulonemal filaments, eventually giving rise to leafy shoots called gametophores. These contain gametangia and, as a result, they are often referred to as gametangiophore. Sexual reproduction is made possible by the development of archegonia (female sex organ) and antheridia (male sex organ) at the apex of the same gametophore (Cove, 2005). Motile spermatozoids that swim to reach the egg in the archegonia for fertilization are formed within antheridia. The resulting zygote develops into a diploid sporophyte which, once fully developed consists of a short stem called seta and a spore capsule. Meiosis occurs within the sporophyte and produces around 4000 haploid spores. After ripening, the spore capsule will rupture and release spores capable of starting fresh mass colonies upon germination (Cove, 2005).

1.5.2 Advantages of *P. patens* in the study of starch metabolism?

The moss *P. patens* is a well suited model system for gene function studies within plants as it is possible to insert DNA into targeted sections of the genome using homologous recombination allowing targeted gene knockouts through the use of a selection cassette. Selection and regeneration of stable transformants is possible within 6-8 weeks. Furthermore, the small size and simple morphology of *P. patens* makes it convenient for cultivation. It is easy to propagate (Prigge & Bezanilla, 2010) and its predominantly haploid life cycle makes it easy to observe recessive traits directly (Engel, 1968; Cove, 2005).

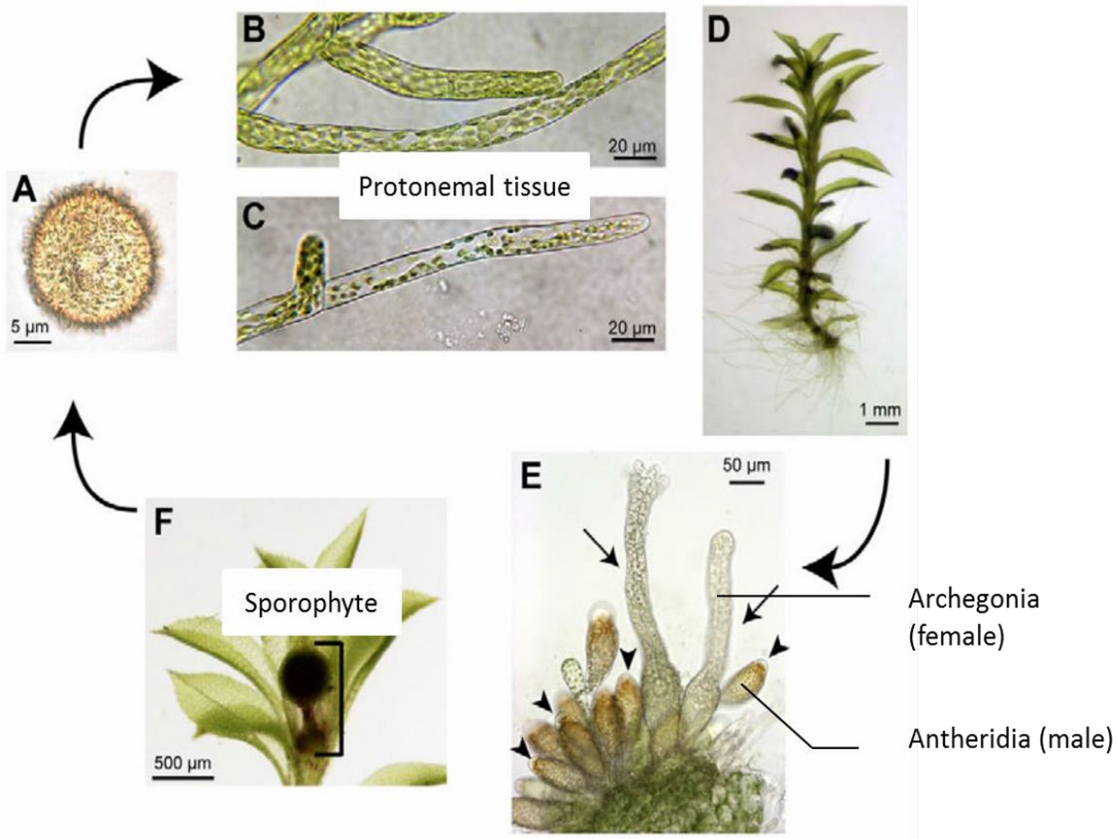

Figure 1.9 The life cycle of *Physcomitrella patens.* A haploid spore (**A**) germinates and produces chloronemal cells (**B**) which eventually differentiate into caulonemal cells (**C**). The leafy gametophores (**D**) emerge from the protonemal tissue (chloronemal + caulonemal cells). At the apex of the gametophore **(E**) both archegonia and antheridia develop. The sporophyte (**E**) is produced as a result of the fertilization of the egg by a motile sperm (Adapted from Prigge and Bezanilla, 2010).

Although much has been learned about the pathway of starch metabolism in *Arabidopsis thaliana* as a model, it is scientifically interesting to examine if this pathway conserved in other species. For example, as discussed above the MEX1 protein appears to transport maltose and glucose in *Arabidopsis*, but only glucose in *Chlamydomonas reinhardtii* indicating an alteration in the pathway since plants colonised land. Investigations into *Physcomitrella* have shown the presence of starch granules (Thornton et al., 2005) and that starch degradation is associated with freezing tolerance (Nagao et al., 2005). In Table 1.3 the numbers of genes potentially involved in starch

metabolism in *Physcomitrella* is shown. Almost all enzyme classes are encoded by more genes than in *Arabidopsis*, most likely due to a gene duplication event that is known to have increased numbers of genes involved in carbohydrate metabolism in *P. patens* (Rensing et al., 2007). This increased number of genes involved in starch metabolism makes the study of this pathway complicated in *Physcomitrella*; nevertheless, the knowledge of the DNA sequences of these genes allows the easy production of knockout mutants. Because of this, and its position as one of the first plants to colonise land, it is an ideal plant to examine if the pathway of starch metabolism has changed since that event.

1.6 Aims and objectives of this study

In this project, I set out to acquire a deeper understanding of starch metabolism in *P. patens* and more specifically the roles of the debranching enzymes GWD, ISA1 and ISA2.The objectives were:

- Examination of the evolutionary relationships between GWD and ISA genes with orthologous genes form other plant species through phylogenetic analysis
- Establishment of the subcellular localisation of proteins encoded by *PpGWD1* like genes through GFP fusion experiments
- Production of *P. patens* lines lacking *PpGWD1, PpISA1* and *PpISA2*expression through inserting a resistance cassette into the respective genes
- Examination of starch and starch phosphate contents in *Ppgwd* mutant lines
- Assessment of growth in *Ppgwd* mutant lines
- Determination of starch and phytoglycogen contents in *Ppisa* mutant lines
- Elucidation of activity of recombinant PpISA1 and PpISA2 protein
- Examination of debranching enzyme activities in *Ppisa* mutant lines

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Chapter Two

Research Article:

The following chapter was published as Mdodana et al. (2019) Scientific Reports 9: 15114. Ms Mdodana was responsible for all experiments and analysis, except for the phylogenetic tree. Detailed author contributions are described at the end of the chapter.

Mutations in Glucan, Water Dikinase Affect Starch Degradation and Gametophore Development in the Moss Physcomitrella patens.

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Abstract

The role of starch degradation in non-vascular plants is poorly understood. To expand our knowledge of this area we have studied this process in *Physcomitrella patens* through examination of the first step of starch degradation, glucan phosphorylation, catalysed by glucan, water dikinase (GWD) enzymes. Phylogenetic analysis indicates that GWD isoforms can be divided into two clades, one of which contains GWD1/GWD2 and the other GWD3 isoforms. These clades split at a very early stage within plant evolution as distinct sequences that cluster within each were identified in all major plant lineages. Of the five genes we identified within the *Physcomitrella* genome that encode GWD-like enzymes, two group within the GWD1/GWD2 clade and the others within the GWD3 clade. Proteins encoded by both loci in

the GWD1/GWD2 clade target to plastids. Mutation of either sequence reduce starch phosphate amounts, however, one isoform had a much greater influence on starch phosphate amounts than the other. Only mutations affecting the isoform with the greatest effect on starch phosphate inhibited starch degradation. Mutants lacking this enzyme also failed to develop gametophores, a phenotype that could be chemically complemented using glucose supplementation within the growth medium.

Introduction

The bryophyte *Physcomitrella patens* is a species that has many advantages for use in the study of plant molecular physiology. Its genome has been sequenced[1], it is transformable, knockout mutations are easily generated through homologous recombination and its generally haploid lifestyle means that mutations can be studied directly in the M_1 generation[2]. *Physcomitrella* and tracheophytes diverged approximately 450 million years ago[1], after which vascular plants developed an increased ability to survive without proximate water sources. During this time, their metabolic pathways will have changed in a way that would advantage vascular plants in the new ecological niches they encountered. The use of this plant to study metabolism allows for the analysis of how biochemical pathways have altered in different plant species since this divergence[3].

We have decided to examine the pathway of starch metabolism in *P. patens*. This is because the presence of this metabolite has been demonstrated to be important for plant growth and development in vascular plants[4,5] and we wish to examine if the same holds true in this nonvascular species. When grown on artificial media, *P. patens* spores or explant material

develop initially into thread like protonemal tissue, containing two distinct cell types, chloronema and caulonema. Caulonemal cells contain fewer chloroplasts than chloronemal cells, and can also form buds which develop into leafy shoots known as gametophores. Many factors are known to affect gametophore development including alterations in cell wall[6], phytohormone synthesis[7,8], light perception[9] and several regulatory genetic elements[10–12]. Alterations in carbon metabolism and sensing also alter colony growth and development alongside influencing starch accumulation[13–15]. Little is known about the role of starch in *Physcomitrella*, although it is present[14,16] and its degradation has been implicated in freezing tolerance[16].

In this study we examine the step that has been shown to initiate starch catabolism in higher plants, starch phosphorylation[17,18]. Much, if not all of the pathway of leaf starch degradation has been recently elucidated through studies in a number of species, primarily *Arabidopsis*, and involves several enzymatic reactions[5,19]. The initial steps occur within chloroplasts where starch is phosphorylated by glucan, water dikinase (GWD) isoforms[20–28] that solubilize the surface of the granule, allowing access to α-amylase, β-amylase, isoamylase and βlimit dextrinase[29–32]. Degradation by these enzymes releases soluble phosphorylated maltooligosaccharides into the stroma. The phosphate from these is removed by two polyglucan phosphatases[33–35] before they can be further degraded to maltose and glucose through the actions of ∝-, β- and isoamylases[29–31] alongside disproportionating enzyme 1[36–38]. Maltose and glucose are exported from the plastid into the cytosol by two transporters[39–41] where the maltose is further mobilized by disproportionating enzyme 2[37,38,42,43].

To help understand the role of starch degradation in *P. patens* we decided to mutate some of the starch phosphorylating enzymes as they catalyse the first step in starch degradation and their importance is, therefore, likely to be conserved between vascular and non-vascular plants. Three of these have been identified in angiosperms: GWD1 is localized to the plastid and phosphorylates amylopectin at the 6-position[20,24]. Mutations in this gene lead to starch without covalently bound phosphate and a large decrease in leaf starch degradation[25,26,44–47]. GWD3 is also plastidial and is often named the phosphoglucan, water dikinase (PWD) as it can only phosphorylate starch that has already been acted upon by GWD1. Mutations eliminating GWD3/PWD lead to starch without phosphate bound at the 3-position and a mild repression of leaf starch degradation[27,28]. GWD2 is present in the cytosol and the gene encoding it is expressed mainly in sieve elements[48]. Although mutations eliminating it do not affect starch turnover in photosynthetic tissue, they affect plant growth[49].

In this study we report on mutations in two GWD1 isoforms and demonstrate that this leads to the synthesis of starch containing reduced glucose 6-phosphate. Mutations in one of the isoforms lead to increased starch accumulation and to colonies that do not produce gametophores.

Results

The *Physcomitrella patens* genome contains multiple GWD isoforms. We examined the presence of sequences encoding GWD-like enzymes within the *Physcomitrella* genome through a tBLASTn search using the *Arabidopsis* GWD1 (NCBI accession NM_001331926.1) amino acid sequence at Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#!search?show=BLAST). This identified five loci on chromosomes 3 (Pp3c3_11200), 8 (Pp3c8_6536), 14 (Pp3c14_19150),

17 (Pp3c17_18900) and 18 (Pp3c18_14870) encoding putative GWD isoforms. BLASTP searches of the *A. thaliana* genome using the predicted amino acid sequence encoded at each loci indicated that the genes on chromosomes 3 and 8 were most similar to AtGWD1 while the other three were most similar to AtGWD3. We decided to name the two genes that appear to encode GWD1 or GWD2 isoforms as *PpGWDa* (Pp3c8_6536) and *PpGWDb* (Pp3c3_11200) while we named the other three loci *PpGWDc* (Pp3c17_18900), *PpGWDd* (Pp3c14_19150) and *PpGWD*e (Pp3c18_14570).

The coding sequences for two of these (*PpGWDa* & *PpGWDb*) are predicted to contain 31 introns, one (*PpGWDc*) contains 6 introns while the final two (*PpGWDd* & *PpGWDe*) are intronless (Fig. 2.1a). Four of the five loci (*PpGWDa, PPGWDb, PpGWDd* & *PpGWDe*) encode proteins containing the histidine known to be involved in transfer of phosphate during the dikinase reaction[50] while the *PpGWD*c locus contains a deletion that eliminates this residue (Fig. 2.1b). PpGWDa and PpGWDb are approximately 1420 amino acids (aa) in length, PpGWDc is 989 aa and the other two approximately 1160 aa. Amino acid sequences encoded at both *PpGWDa* and *PpGWDb* loci contain the CFATC motif thought to be involved in redox regulation[51], while in PpGWDd and PpGWDe that motif is altered to VFVTC. The deletion present within *PpGWDc*, which eliminates the catalytic histidine, also removes the VFVTC containing region (Fig. 1.1b). All polypeptides encoded by these loci contain C-terminal pyruvate phosphate dikinase or PEP synthase domains. PpGWDa and PpGWDb contain N-terminal PLN02784 α-amylase domains, whilst CBM20 domains are present at the N-termini of PpGWDc, PpGWDd and PpGWDe (Fig. 2.1c).

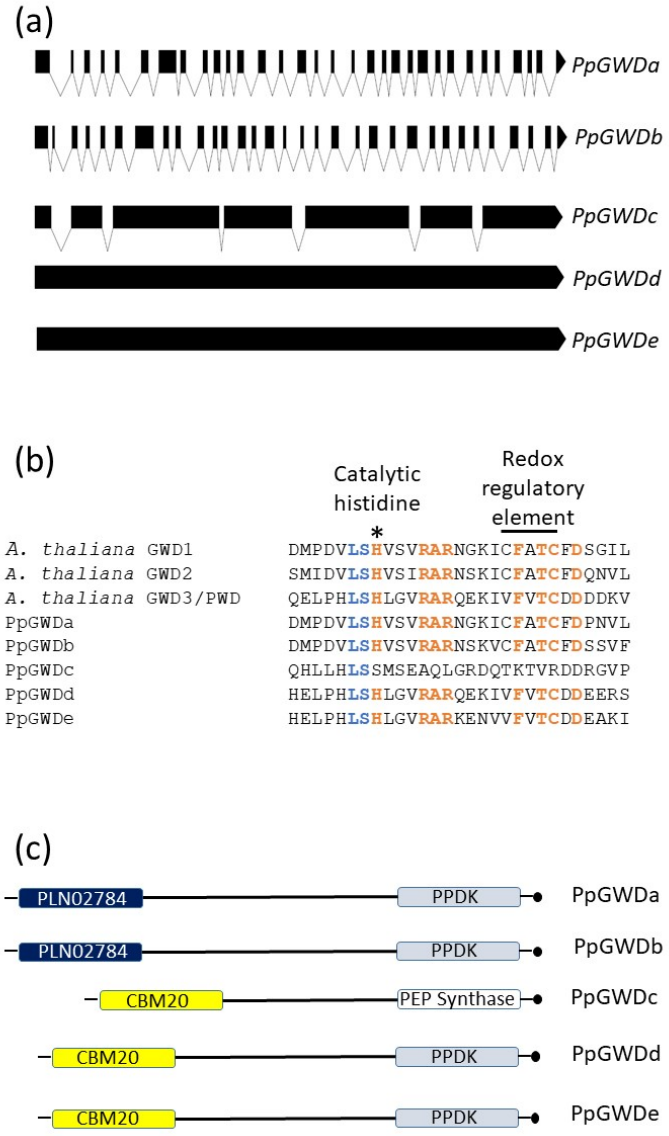
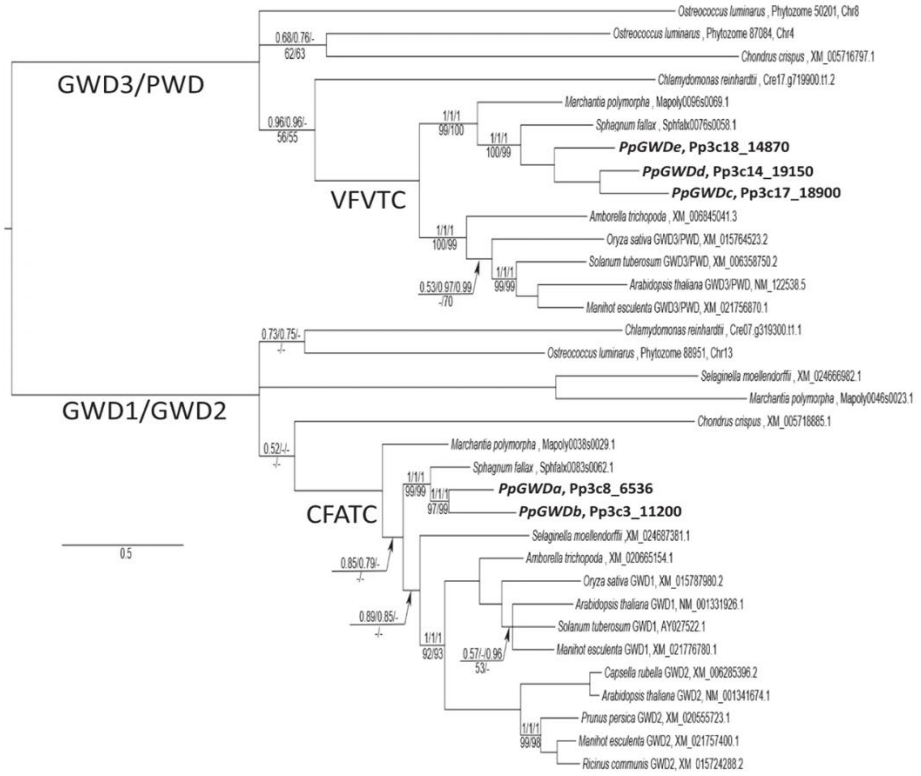


Figure 2.1. Analysis of putative GWD encoding sequences from *Physcomitrella*. (a) Predicted exonintron structure of the five identified loci. (b) Amino acid sequence within the active sites of the predicted proteins in comparison with those from *Arabidopsis*. (c) Domain structure within the PpGWD proteins. PLN02784, CBM20, Pyruvate phosphate dikinase (PPDK) and PEP synthase domains are shown at the approximate sites that they are found within the polypeptides. The length of the figures represent the relative number of amino acids (aa) present in PpGWDa (1420aa), PpGWDb (1415aa), PpGWDc (989aa), PpGWDd (1170aa) and PpGWDe (1148aa). The black circle denotes the C-terminus.

A recent phylogeny of GWD sequences has been published[47], but this only included three of the five *Physcomitrella* sequences and none from other non-vascular plants. To examine the relationships of the five sequences identified from the *Physcomitrella* genome with other GWD genes and proteins we have produced new phylogenetic trees including all *Physcomitrella* sequences as well as others from a number of red and green algae, non-vascular and vascular plants. Analysis was performed using both nucleotide (DNA; Fig. 2.2a) and amino acid (AA; Fig. 2.2b) data in both a maximum likelihood (RAxML) and Bayesian (MrBayes) context.

All MrBayes runs reached stationarity (all Potential Scale Reduction Factors between 0.99 and 1.01) and achieved adequate sample sizes (minimum ESS across combined runs > 400). MrBayes AA runs overwhelmingly (posterior probability = 1) chose the WAG model[52] as the bestfitting empirical model, which was used for RAxML AA analyses. When rooted on the longest internal branch, the tree consisted of two major clades, one containing GWD3/PWD sequences, and the other containing all GWD1 and GWD2 sequences. The five *Physcomitrella* sequences grouped in two distantly-positioned portions of the tree. The first position, retrieved in all analyses with near-maximal support, contained the three intronless/intron-poor sequences as each other's closest relatives, with *Sphagnum fallax* (Sphfal0076s0058.1) and *Marchantia polymorpha* (Mapoly0096s0069.1) as successive sisters in all analyses with maximal or nearmaximal support. All five bryophyte sequences grouped in a land plant clade with maximal support; these land plant sequences also uniquely shared the VFVTC sequence. The second position contained the two intron-rich sequences as sister to *Sphagnum fallax* (Sphfal0083s0062.1) with strong support (DNA) and moderate to strong support (AA) in all

(a)



(b)

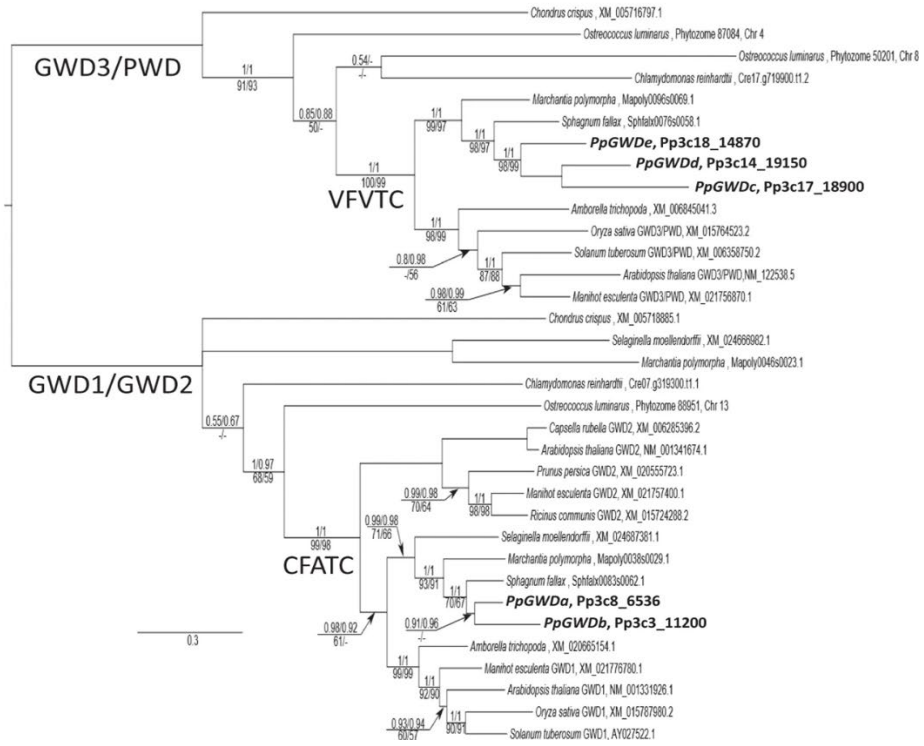


Figure 2.2. Phylogeny of GWD sequences from various plant species. (a) : 50 % majority rule consensus tree from partitioned MrBayes analysis of the full DNA data set of Archaeplastid GWD/PWD sequences, showing phylogenetic placement of determined *P. patens* (bold) paralogs. The three numbers listed above branches are Bayesian posterior probabilities (PP) for the full, partitioned analysis, PP for a partitioned analysis with a region of uncertain homology excluded, and PP for the recoded RY analysis. Numbers under each branch are RAxML likelihood bootstrap support (BS) for the full, partitioned analysis and BS for a full, partitioned analysis with a region of uncertain homology excluded. Dashes indicate support for a branch < 50 %, or where the branch was not present in a particular analysis. Un-numbered branches received maximal support in all analyses. The scale bar is in substitutions per site. (b) 50 % majority rule consensus tree from partitioned MrBayes analysis of the full AA data set of Archaeplastid GWD/PWD sequences, showing phylogenetic placement of determined *P. patens* (bold) paralogs. The two numbers listed above branches are Bayesian posterior probabilities (PP) for the full analysis, and PP for analysis with a region of uncertain homology excluded. Numbers under each branch are RAxML likelihood bootstrap support (BS) for the full analysis and BS for analysis with a region of uncertain homology excluded. Dashes indicate support for a branch < 50 %, or where the branch was not present in a particular analysis. Unnumbered branches received maximal support in all analyses. The scale bar is in substitutions per site. In (a) and (b) the branches subtending two land plant clades characterized by specific motifs are labelled with the corresponding amino acid sequence (VFVTC and CFATC). Sequences are identified either by Phytozome AGI codes or NCBI accession numbers.

analyses. DNA- and AA-based analyses differed on the closest relatives of this moss clade, with DNA analyses equivocal and AA analyses supporting *Marchantia polymorpha* (Mapoly0038s0029.1) and *Selaginella moellendorffii* (XM_024687381.1) as successive sisters, with moderate to strong support. These sequences formed part of a strongly supported land plant clade which shared the CFATC redox regulation motif (or minor variants thereof). In addition to the bryophyte/*Selaginella* sequences, this land plant clade included a clade containing all characterized GWD1 accessions, and a last clade containing all characterized GWD2 accessions, but relationships among these differed.

Both GWDa and GWDb localise to the plastid. We examined the presence of transit peptides in all five genes using the ChloroP server. PpGWDa, b and e were predicted to target to plastids, while PpGWDc and d were predicted not to contain transit peptides. To confirm the localization of the GWD1 like isoforms we produced constructs where cDNA of either gene was fused in frame with GFP at the C-terminus. These were transformed into *P. patens* protoplasts and transiently expressing samples imaged by confocal microscopy. In both cases the GFP signal coincided exactly with that of chlorophyll, demonstrating that both proteins are plastidial (Fig. 2.3a).

Mutations in both genes affect starch phosphorylation. We used homologous recombination to manufacture mutants. The constructs were designed to replace DNA encoding the known dikinase domain from either gene (Fig. 2.2a, Supplementary Fig. 2.1), which includes the catalytically essential histidine residue[50], with a disruption cassette flanked with *loxP* sites. For each gene we recovered two plants that grew on hygromycin following transformation with gene disruption cassettes, and named these *Ppgwda*-1or –2 and *Ppgwdb*-1 or –2. We also removed

the disruption cassettes from both lines containing inserts at the *PpGWDa* locus using cre recombinase and, in these lines, mutated the *PpGWDb* locus to produce two double mutant (DM-1or -2) lines.

To confirm that the lines we created were mutated, PCR analysis was performed using gDNA template and two sets of primers (Fig. 2.3b). One primer set, which binds either side of the homologous recombination sites, was used to detect the presence of either wild type alleles (amplicon sizes of 4500bp for *PpGWDa* and 6000bp for *PpGWDb*) or deletions within the *PpGWDa* locus in the DM (amplicon size approximately 700bp). This demonstrated that wild type *PpGWDa* alleles were present in the *Ppgwdb* mutants and wild type *PpGWDb* alleles were present in *Ppgwda* mutants. On the other hand, in both DM lines a mutant *Ppgwda* allele was present that contains the deletion leading to loss of the catalytically essential histidine. A second primer set was used where one primer binds upstream of the insertion and another within the disruption cassette. This demonstrated the presence of the cassette within the *PpGWDb* locus in both single and double mutant lines (Fig. 2.3b). As expected the PpGWDa amplicon in the DM was approximately 500bp due to the elimination of the resistance cassette and part of the gene. We attempted to examine protein amounts using a GWD antibody that was raised against the potato protein[25], but it did not recognize any *Physcomitrella* GWD polypeptide. Therefore, we examined gene expression using semi quantitative RT-PCR and showed that RNA transcribed from either *PpGWDa* or *PpGWDb* loci was abolished whenever the appropriate gene disruption cassette was present (Fig. 2.3c and Supplementary Fig. 2.2).

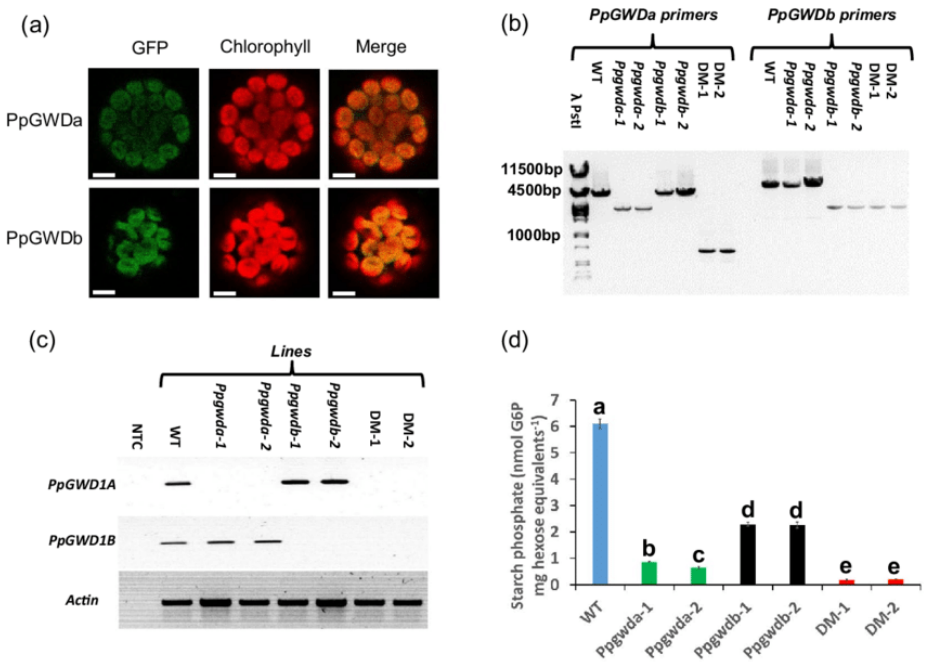


Figure 2.3. Examination of GWD in *P. patens.* (a) Protoplasts expressing PpGWDa or PpGWDb fused to GFP. Scale bar is 5 μm. (b) PCR analysis from gDNA demonstrating the presence of mutant alleles in *Ppgwda*, *Ppgwdb* or double mutant (DM) lines. Amplicons were separated on a 1% (w/v) agarose gel. λ-PstI represent λ phage DNA digested with PstI. (c) Semi-quantitative RT-PCR analysis of *PpGWD1a*, *PpGWD1b* or *Actin* expression in the wild-type (WT), *Ppgwd1a*, *Ppgwd1b* and DM experimental lines. NTC designates the no template control. Original gels are shown in Supplementary Fig. 2.1. (d) Glucose 6-phosphate amounts in starch from the wild-type and mutant lines. Data represents means of three independent digestions of pooled starch samples ± SEM. Letters represent groups with similar means at the 5% significance level as determined using the Bonferroni-Holm *post hoc* test following a one-way analysis of variance.

As GWD isoforms are known to phosphorylate starch in vascular plants, we examined the amounts of covalently bound starch phosphate in both the single and double mutants (Fig. 2.3d). There was a significant decrease in all the mutant lines. Starch from *Ppgwda* mutant lines contained approximately one seventh of the glucose 6-phosphate of the control, while lines containing an insert within the *PpGWDb* locus contained approximately one third. Glucose 6phosphate was reduced below that of the *Ppgwda* lines in the double mutants.

Mutations in *PpGWDa affect* starch degradation. To examine starch degradation in the mutant lines we grew colonies for 8 weeks growth on artificial medium before determining starch and soluble sugars over a diurnal cycle from entire colonies (Fig. 2.4). Starch contents increased during the light period and decreased during the dark. The WT and *Ppgwdb* mutants accumulated similar amounts of starch at all time-points as did the *Ppgwda* and DM mutants. Starch was significantly reduced ($p \leq 0.05$) in the WT and *Ppgwdb* lines in comparison with *Ppgwda* and DM mutants at almost all time-points. The one exception was at 0 hours in the second set of mutants when the starch content in the WT and the *Ppgwda* mutant were invariable. Conversely, soluble sugars were reduced in the *Ppgwda* and DM mutant lines compared with the WT and *Ppgwdb* mutant. Within both mutant sets glucose was significantly ($p \leq 0.05$) reduced in the *Ppgwda* and DM lines compared with the others at the 0, 8, 16 and 20 hour time points. Similarly fructose was significantly ($p \leq 0.05$) reduced at 20 and 24 hours and sucrose at 0, 8, and 20 hours.

***Ppgwda* and DM plants demonstrate altered colony morphology.** There were no consistent significant differences in growth between the lines when grown on BCD medium, however, we noted alterations in colony morphology with the WT and *Ppgwdb* lines developing gametophores

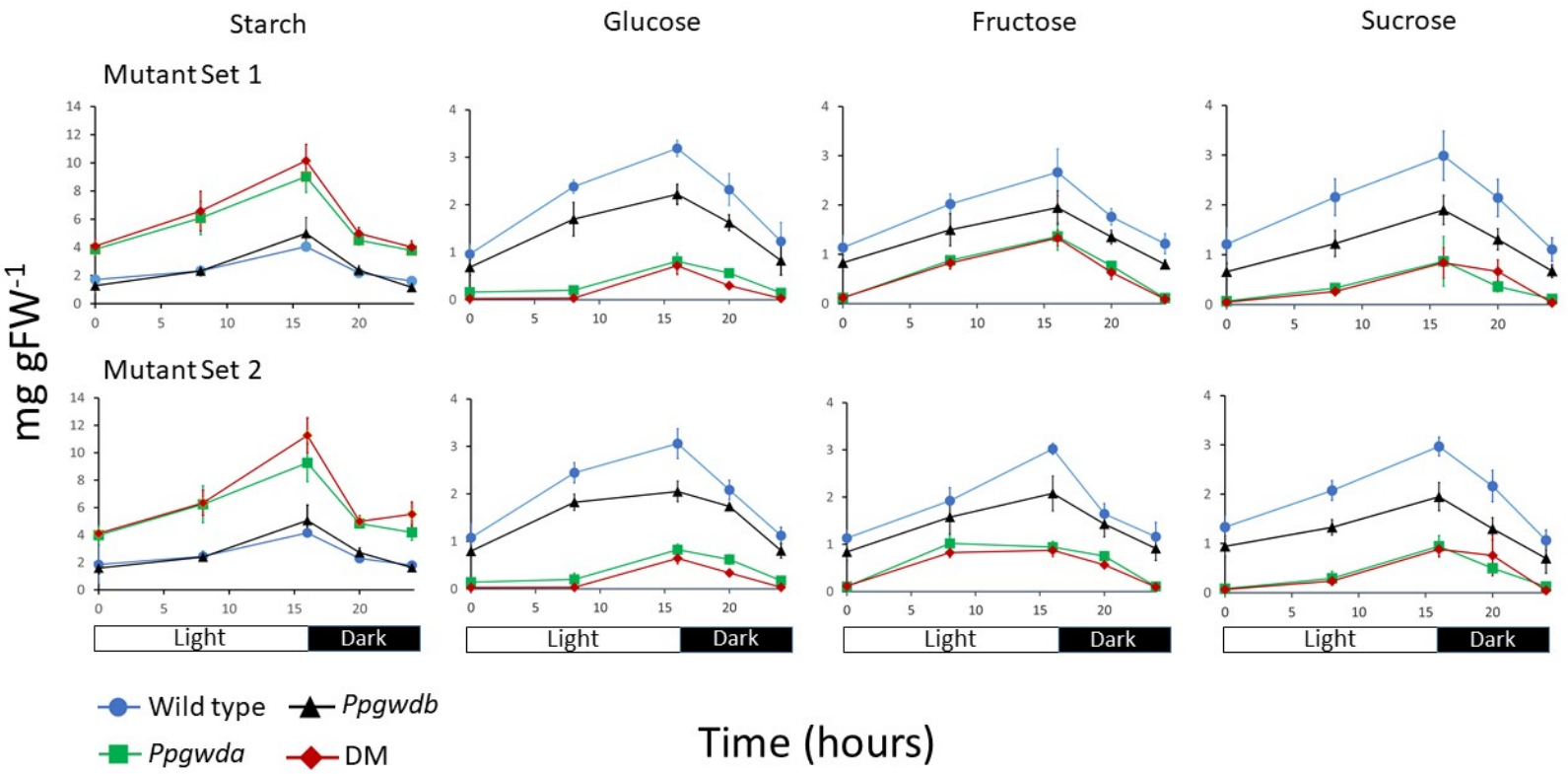


Figure 2.4. Starch and soluble sugar amounts in colonies from the WT and mutant lines. Plants were grown on BCD medium for 8 weeks under a 16h/8h day/night regime. Tissue was sampled at five time points over a 24 hours period and starch, glucose, fructose and sucrose determined. Data represents means of at least 5 colonies. Error bars are SEM and, if not visible, are within the symbol.

while the *Ppgwda* and DM lines did not. Data from the first mutant set are presented in Figure 4 and 5, while data from the second set are presented in Supplementary Figs. 3 and 4. Colonies from the wild type and *Ppgwdb* mutants contained significantly ($p<0.05$) increased numbers of gametophores compared with *Ppgwda* and DM lines at all time points, when grown on BCD media or BCD media supplemented with mannitol. When the plants were grown on media containing glucose all lines grew faster and all produced significant numbers of gametophores (Fig. 2.5 & Supplementary Fig. 2.3). The *Ppgwda* and DM lines still contained significantly fewer gametophores ($p<0.05$) than the WT at weeks 1 and 5, but at weeks 2, 3 and 4 they contained similar numbers (Fig. 2.5 and Supplementary Fig. 2.3).

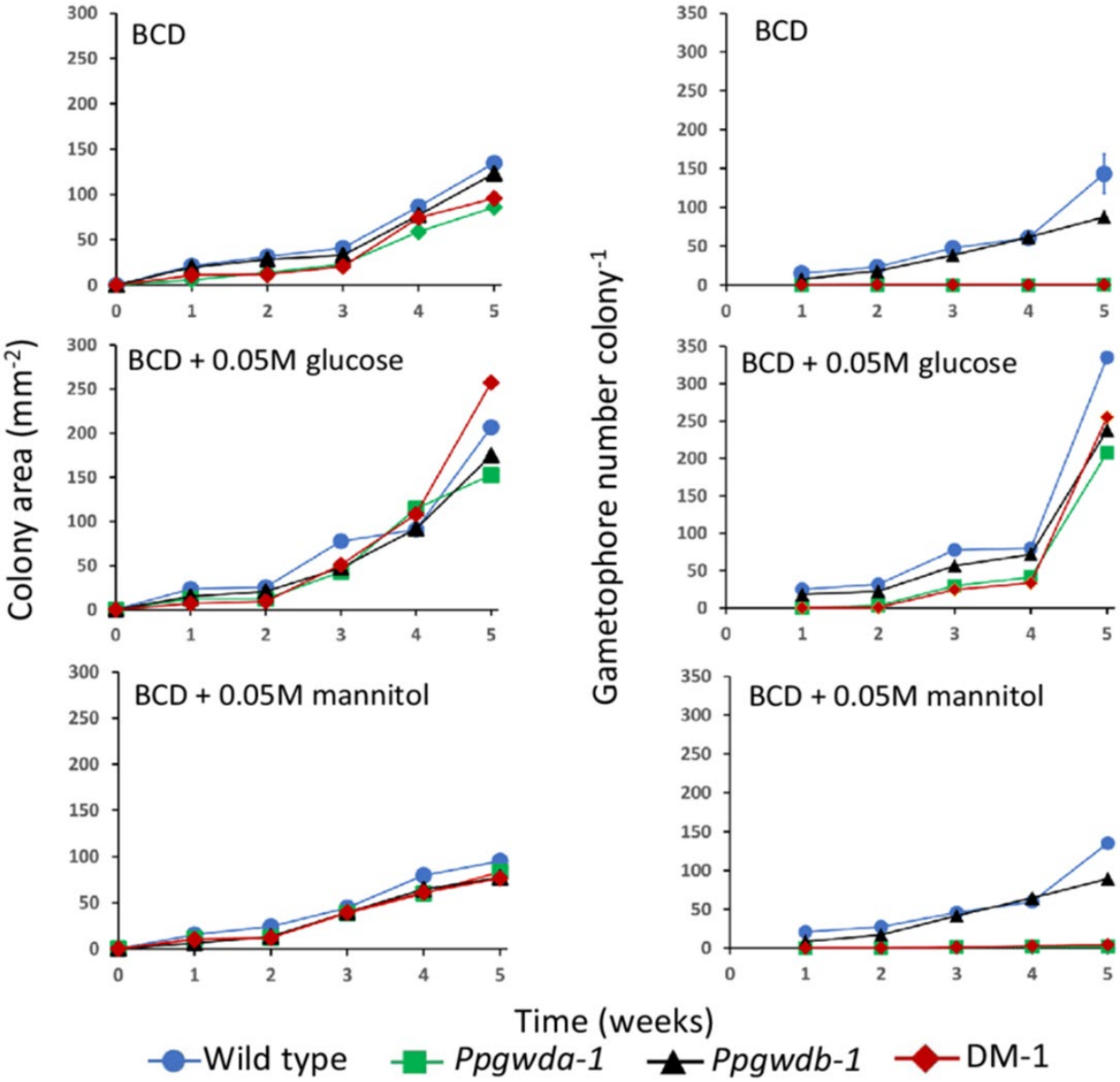


Figure 2.5. Growth and gametophore number in the α-mutant lines. Colonies were established on BCD media, BCD + 0.05M glucose and BCD + 0.05M mannitol and allowed to grow for 5 weeks. Data represents means of at least 3 colonies. Error bars are SEM and, if not visible, are within the symbol.

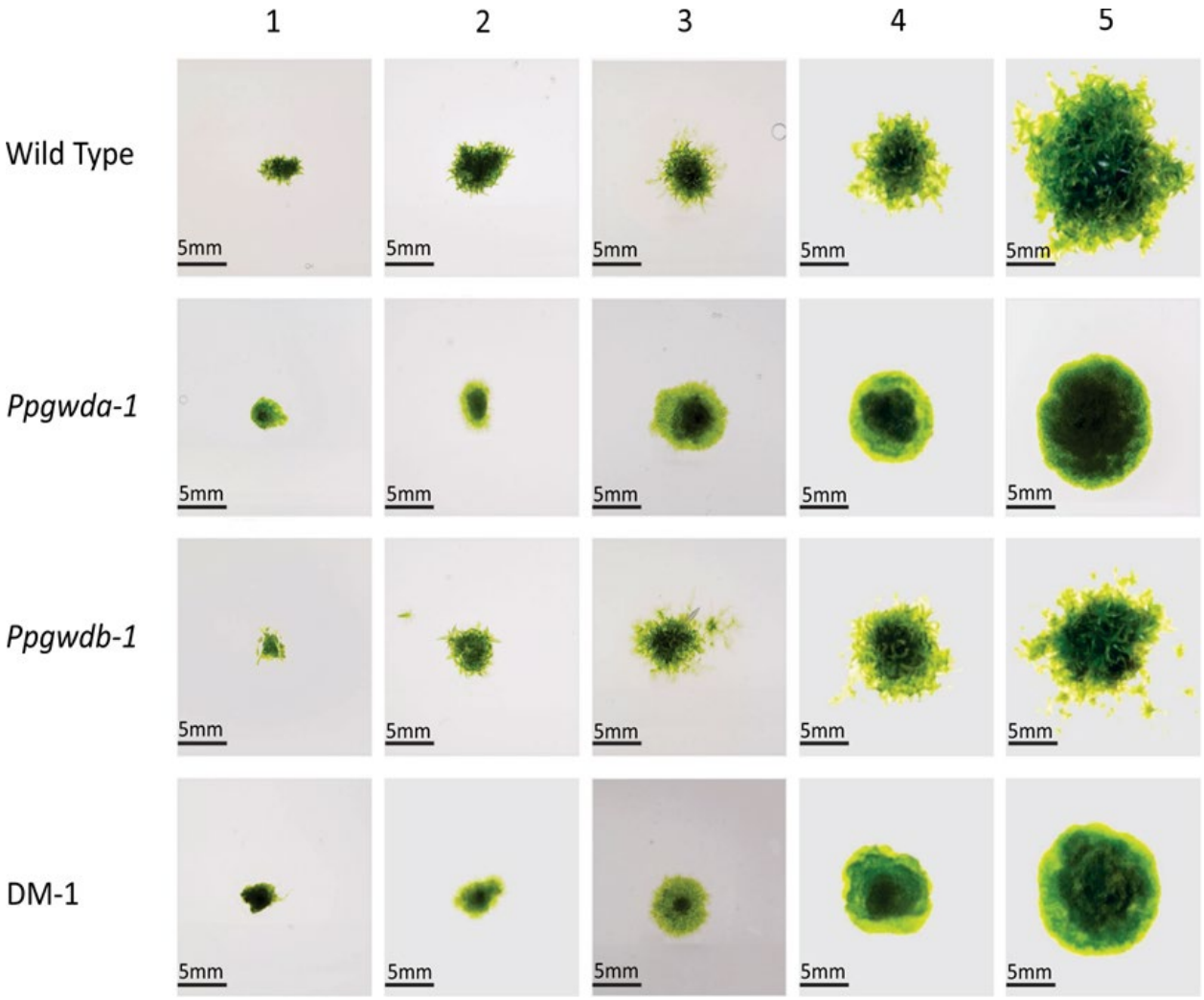


Figure 2.6. Colony morphology of wild-type and α mutant lines grown on BCD medium for 1, 2, 3, 4 and 5 weeks. Scale bar = 5mm.

Discussion

Starch degradation is important for the normal growth and development of some angiosperms[5], but knowledge about its role in these processes in non-vascular plants is lacking. Some work has been initiated in *Chlamydomonas* where a forward genetic approach has identified mutants affecting starch catabolism[53], but to date only plants mutated in the plastidial maltose transporter have been characterized[54,55]. To broaden our knowledge of this process in nonvascular plants we have examined the role of GWD1 like enzymes in *Physcomitrella patens* through production of targeted mutants.

Analysis of the *Physcomitrella* genome identified five genes encoding proteins with significant similarity to GWD1 from *Arabidopsis* (Fig. 2.1). Phylogenetic analyses (Fig 2.2a, b) demonstrated the presence of two clades, each containing red algal, green algal and land plant representatives in an approximate recapitulation of recognized Archaeplastid phylogeny. This indicates that the duplication leading to GWD3/PWD-type genes and GWD1/GWD2-type genes was present in the last common Archaeplastid ancestor; yet we could find no clear homologs to any GWD-type gene in *Gloeomargarita lithophora*, the recently identified cyanobacterium thought to be sister to the plastid endosymbiont[56]. Subsequent duplications led to different isoforms of *Ostreococcus*-type GWD3/PWD genes, an uncharacterized GWD1/GWD2-type lineage shared by *Selaginella* and *Marchantia*, and the separation of GWD1 and GWD2 clades somewhere between the evolution of tracheophytes and angiosperms.

The differing topologies in the DNA/AA phylogenies suggest two potential evolutionary scenarios: the DNA-based trees (Fig. 2.2a) indicates a duplication event leading to the formation

of GWD2 isoforms after the divergence of bryophytes and tracheophytes i.e. acquisition of GWD2 function and localization from a GWD1-type ancestor. Alternatively, the AA trees (Fig. 2.2b) suggest a much deeper separation of GWD1/GWD2 lineages, at or near the origin of land plants, but with GWD2 genes either deleted from or undetected in all non-angiosperm land plants, and ancestral function uncertain. Our analysis indicates that there are two *P. patens* genes (*PpGWDa* and *PpGWDb*) encoding either GWD1 and/or GWD2 isoforms. Like AtGWD1[26], but unlike AtGWD2[48], both encode polypeptides that are targeted to plastids (Fig. 2.3a). This may indicate that the interpretation from the DNA tree is the correct one, but similar analysis of GWD1/GWD2 like isoforms in green algae, other non-vascular plants, seedless vascular plants and gymnosperms will be needed to confirm this. The other three genes are most likely GWD3/PWD isoforms and they group in that clade and all contain CBM20 motifs that are found in GWD3/PWD, but not GWD1 isoforms[17] (Fig. 2.1c). Interestingly the two *PpGWD1/GWD2* genes contained similar exon intron structures, while *PpGWDd* and *PpGWDe* were intron less (Fig 1a). All four of these genes encode proteins predicted to contain the known active site and elements identified in vascular plant GWD1's as redox regulatory, or variants of them (Fig. 2.1b)[50,51]. These GWD1/GWD2 and GWD3/PWD gene pairs were most likely formed during the recent genome duplication event that is thought to have occurred in *Physcomitrella* 30-60 million years ago, and which is known to have led to increased numbers of genes involved in metabolic processes[57]. *PpGWDc* contains 7 exons, but also a large deletion, meaning that it encodes a protein significantly smaller than the others which lacks the catalytically essential histidine[58] (Fig. 2.1) meaning that it is unlikely to be active.

To functionally examine the roles of the PpGWD1/PpGWD2 like enzymes we manufactured mutants in each gene as well as isolating double mutants (DM) lacking both (Fig. 2.3b, c). The homologous recombination constructs used to produce the mutants were designed to remove parts of the genes encoding the known active site of the protein (Fig. 2.2b; Supplementary Fig. 2.1) meaning that any RNA manufactured from the remaining gDNA would encode an inactive polypeptide. The inserts would, however, potentially allow expression of RNA from gDNA upstream of the insert sites, which includes the PLN02784 glucan binding domains. We cannot, therefore, rule out the possibility that protein produced from this could affect starch metabolism in some way. We feel that this is unlikely as point mutation in the *AtGWD1* gene allow production of inactive protein containing the starch binding domain, but demonstrate a similar phenotype as knockouts[26]. For both genes we produced two independent sets of single or double mutants. We examined if the mutations reduced starch bound phosphate and found that insertions in either gene reduced the amount of glucose 6-phosphate in starch (Fig. 2.3d). The reduction was greater when the insertion was present in the *PpGWDa* locus than when *PpGWDb* was mutated. Starch phosphate content in the double mutants was, however, lower than either single mutant line. This demonstrates that both genes encode proteins that incorporate phosphate at the 6-position of glucose moieties within the starch polymer *in vivo*, the known biochemical function of GWD1 isoforms, and that PpGWDa plays a greater role in this process than PpGWDb.

Reduction in starch phosphate caused by mutations in GWD1 has been demonstrated to impair starch catabolism in angiosperms[25,26,45,46]. This is believed to be due to the phosphate disrupting double helices between amylopectin chains and allowing access to amylases to initiate

starch granule degradation[17,18]. To examine this we grew plants over a diurnal cycle and examined starch and soluble sugar levels (Fig. 2.4). As in angiosperms starch increased during the light period and decreased when it was dark. Both *Ppgwda* and DM lines contained significantly more starch than the WT or *Ppgwdb* line. Conversely, soluble sugar contents were reduced at most time points in the DM and *Ppgwda* mutants compared with the WT and *Ppgwdb* lines (Fig. 2.4). This demonstrates that mutations affecting PpGWDa reduce starch degradation in *P. patens* as *Ppgwda* and DM mutant plants develop a starch excess phenotype, but that PpGWDb has little effect on this process. Although the plants lacking PpGWDa develop a starch excess phenotype, they can still degrade starch. Indeed they degraded more starch between time-points than either the WT or *Ppgwdb* (Fig. 2.4) most likely due to the larger amounts of starch they accumulate prior to the start of the experiment. This is similar to other plants lacking GWD where starch is still degraded in leaves over a diurnal cycle, but where a starch excess phenotype develops over time due to small differences in the amount of starch that is mobilised at night[25,26,46].

The influence of the two enzymes on starch catabolism is in line with their observed effects on starch phosphate amounts where PpGWDa has a greater influence than PpGWDb (Fig. 2.3d). However, the relationship between starch phosphate reduction and starch catabolism is not linear as mutations in PpGWDb reduce starch phosphate by more than 50%, but do not affect starch degradation. This indicates that starch phosphate must be reduced below a threshold level before an effect on starch degradation is observed and that the starch phosphate is above that threshold in the WT and *Ppgwdb* mutants, and below in the *Ppgwda* and DM lines. Interestingly a previous study[59] in *Arabidopsis* demonstrated that GWD amounts had to be reduced below a

threshold level before starch degradation was inhibited, although the authors did not examine starch phosphate in that study.

Mutant plants lacking PpGWDa demonstrated an altered appearance when grown on BCD medium, with both *Ppgwda* and DM plants developing almost no gametophores (Figs. 2.5 and 2.6 and Supplementary Figs. 2.3 and 2.4). We hypothesized that the decrease in starch degradation would reduce soluble sugars within plant cells, and that this could impact both growth and plant development. To examine this, we grew plants on media containing either 0.05M glucose or 0.05M mannitol. When placed on glucose-containing medium, all plants grew more quickly and both *Ppgwda* and DM lines developed gametophores, although not to the same extent as wild type or *Ppgwdb* mutants. Neither the increase in growth nor gametophore development occurred when *Ppgwda* or DM lines were grown on mannitol-containing medium, indicating that the reversion of the mutant phenotype is caused by glucose uptake and not the altered osmotic potential. Interestingly mutations in *PpGWDb* also reduced gametophore number to about two thirds of the wild-type strain. It is not clear why this is the case as we could not identify decreased starch degradation in these lines. It is possible that *PpGWDb* is present at specific growth stages or in only some cell types leading to a localized reduction in starch degradation that impacts on gametophore development.

These data demonstrate that starch-derived soluble sugars influence the development of gametophores in *P. patens*. It is possible that decreased soluble sugars inhibit gametophore development directly due to reduction in carbon skeletons, possibly through decreasing substrate supplies to a cellulose synthase which are essential for gametophore development[6]. It

is, however, also possible that downstream processes repressing gametophore initiation are affected by the reduced sugar levels. The development both of gametophore forming caulonemal tissue and gametophores is known to be under hormonal control, being influenced by auxins[7] and cytokinins[8], as well as a recently identified ancestral gibberrelin[60]. It is unclear if amounts of any of these hormones have been altered to lead to this phenotype, although it is interesting to note that mutations affecting starch degradation in higher plants affects gibberellin synthesis[61]. Our future work will examine alterations in these factors within the *Ppgwd1a* mutant to identify the underlying changes that inhibit gametophore formation.

Methods

Analysis of *GWD* genes from *P. patens* and comparison with those from other species. tBLASTn searches of the *Physcomitrella patens* genome were performed using the GWD1 sequence from *Arabidopsis thaliana* (UniProtKB# Q9SAC6) at Phytozome 12.1.6. Genomic sequences of putative GWD encoding genes were downloaded and predicted exon-intron boundaries were visualized at www.wormweb.org/exonintron. The presence of predicted chloroplast transit peptides was examined using ChloroP 1.1[62] (http://www.cbs.dtu.dk/services/ChloroP/) and conserved domains within predicted polypeptides were identified through searches of the CCD database[63] at https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi.

DNA sequences encoding GWD polypeptides were obtained from either the NCBI or Phytozome and used to construct a phylogeny. DNA encoding the predicted PPDK domains from *GWD* were translated to amino acid sequence and aligned using Muscle[64] in MegaX[65]. This was followed by subsequent manual alignment of clearly homoplasious indels, as well as of a region encoding

amino acids 214-304 from the start of the alignment, which was highly variable. Phylogenetic analyses were conducted using the CIPRES[66] implementation of MrBayes 3.2.6[67] and RAxML v8.2.4[68], using both the nucleotide (DNA) and amino acid (AA) alignments. All DNA analyses were partitioned by codon position, which yielded vastly improved log likelihood values over naïve analyses (mean Δln likelihood values MrBayes: > 1000; Δln likelihood values RAxML: > 540). MrBayes partitions were allowed to average over GTR submodels (lset nst=mixed) and assigned a gamma correction for among-site rate variation. All MrBayes analyses were run twice, with 1 million generations and default burnin fractions. All runs were checked for stationarity and adequate estimated sample sizes using MrBayes' own diagnostics and the program Tracer v1.61.7 [69]. RAxML DNA partitions were individually assigned the GTRGAMMA model under otherwise default settings, with the bootstopping criterion to determine adequate bootstrap replicates[70]. In addition, because of > 35 % difference in GC content across sampled sequences, which can bias phylogenetic inference[71], nucleotide alignments were also transformed to RY-coding (purine-pyrimidine coding) to check for artefacts introduced by base composition heterogeneity and run in MrBayes under the previously mentioned parameters. AA alignments in MrBayes were allowed to average over a set of empirical substitution rate matrices (prset aamodelpr=mixed), with a gamma correction applied. Otherwise MrBayes AA analyses were run under the same settings and checked in the same way as the nucleotide results. RAxML AA results used the bestfitting empirical matrix from the MrBayes runs as default, and otherwise were run as for the DNA results. Finally, both DNA and AA matrices were run excluding the highly variable region as defined above, to check whether this region of uncertain homology unduly influenced results. All

trees were rooted on the longest internal branch, which was also the branch chosen by midpoint rooting.

Plant material and growth conditions. *P. patens* ssp*. patens* strain Gransden was a kind gift of Dr. P. Hills (Institute for Plant Biotechnology, Stellenbosch University, South Africa). Standard growth conditions for cultures were 23±2°C with a light intensity of 50 µmol photons m^{-2} s^{-1} (Osram L 58V/740, Germany) and a 16/8 hour light/dark regime. Cultures used for starch phosphate analyses were grown on $PpNH_4$ medium (1mM $MgSO_4$, 1.85 mM KH_2PO_4, 10 mM KCL, 45 mM $FeSO_4$, 1 mM $CaCl_2$, 9.93 µM H_3BO_3, 0.103 µM Na_2MoO_4, 0.266 µM $CoCl_2$, 0.191 µM $ZnSO_4$, 1.97 µM $MnCl_2$, 0.169 µM KI, 0.22 µM $CuSO_4$, 0.57 µM $Al_2(SO_4)_3$, 5mM diammonium tartrate) supplemented with 1% (w/v) sucrose. For analysis of growth rates and plant morphology, colonies were divided into approximately $2mm^2$ pieces and placed on BCD media (1mM $MgSO_4$, 1.85 mM KH_2PO_4, 10 mM KNO_3, 45 mM $FeSO_4$, 1 mM $CaCl_2$, 9.93 µM H_3BO_3, 0.103 µM Na_2MoO_4, 0.266 µM $CoCl_2$, 0.191 µM $ZnSO_4$, 1.97 µM $MnCl_2$, 0.169 µM KI, 0.22 µM $CuSO_4$, 0.57 µM $Al_2(SO_4)_3$) or the same media supplemented with either 0.05M glucose or 0.05M mannitol.

Silencing construct preparation. Genomic DNA was extracted from *P. patens* by the protocol of Edwards *et al*[72]. Using this as template, amplicons encoding sections of glucan, water dikinase (*PpGWD1a* or *PpGWD1b*) genes were amplified by PCR with gene specific primers (GWDa FWD 5'TTCAGCCAGATAGCGTCGTC3' and GWDa REV 5'GCCATGCACAAGTCGCTATG3'; GWDb FWD 5'TTGCAGGCGGCTTCTGAACTA3' and GWDb REV 5'GCTCCCACAAGTGTCTCTCC3'). Amplicons were subsequently cloned into the pJET1.2/blunt vector using a CloneJET PCR Cloning Kit, according to manufacturer's specifications (Thermo Scientific, Waltham, MA, USA). The plasmid containing

PpGWDa was digested with SalI and SphI before a loxP-flanked Hygromycin B phosphotransferase (*hph*) resistance cassette was excised from the pMBLH8a[73] plasmid by digesting with the same enzymes and ligating into the genomic =fragment. The plasmid containing the *GWDb* genomic fragment was restricted using StuI and ClaI and the same *hph* resistance cassette was excides using EvoRV ClaI fragment from pMBLH8a was ligated between into the *GWDb* genomic DNA. The resulting mutations constructs (PpGWDa-HygR-KO and PpGWDb-HygR-KO) were digested with either Not1 (PpGWDa-HygR-KO) or XbaI (PpGWDb-HygR-KO) restriction enzymes to produce linear plasmids for transformation.

Production of single and double mutants. Protoplasts were transformed using PEG mediated transformation and mutant plants recovered following selection[74]. For production of double mutants the disruption cassette in the *Ppgwda* single mutant was removed using Cre recombinase. The DNA sequence encoding Cre-recombinase was amplified by PCR from pMM23[75] using the following primers: Cre Forward 5'ATGTCCAATTTACTGACCGTAC3' and Cre Reverse 5'CTAATCGCCATCTTCCAGC3'. Purified amplicon DNA was cloned into the pCR8/GW/TOPO according to the manufacturers (Life Technologies, USA-CA) specifications. The expression cassette from pBinAR-Hyg[76] was excised using the restriction enzymes EcoRI and HindIII and ligated into the same sites within pCAMBIA2200 (http://www.cambia.org/daisy/cambia/585). A Gateway reading frame cassette (Gateway Vector Conversion Kit, Thermo Fisher Scientific) was ligated in the SmaI site of the expression cassette polylinker in sense orientation with respect to the 35S promoter. The cre-sequence was transferred into this vector using LR clonase (Life Technologies®) as described in the

manufacturer's instructions to obtain pCAMBIA2200-Cre. Protoplasts isolated from *Ppgwda* mutant lines were transformed with pCAMBIA2200-Cre using PEG[74]. Protoplasts were regenerated on BCDAT (BCD medium containing 5Mm ammonium tartrate) medium lacking antibiotics for two weeks, after which they were transferred to BCDAT containing 50 µg/mL geneticin. After one 1 week, colonies that had survived the selection stage were divided into two, and plated on both selective (BCDAT plus Hygromycin-B) and non-selective (BCDAT) media and allowed to grow for another week. Replicates growing only on non-selection medium were screened for loss of *hph* resistance cassette. Plants confirmed as lacking the disruption cassette and containing the deletion within *PpGWD1a* were further mutated using PpGWD1b-HygR-KO using the same method as for the production of single mutants.

Semi-quantitative reverse-transcriptase PCR. Total RNA was extracted from 2 week old *P. patens* protonemal tissue using Qiagen RNeasy® Plant Mini Kit (Whitehead Scientific, Cape Town, South Africa), according to the manufacturer's instructions. Synthesis of cDNA was performed with the purified RNA by using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). The following primers were used in the PCR: GWD1A sqRTFwd 5'ACTTGTGCATGGCAGTGTT3' GWD1A sqRTRev 5'AACGACGTACAGTTCACCATC3', GWD1B sqRT Fwd 5'GTGGATCCGTCTTCCAACAT3' GWD1B sqRTRev 5'AATATGCTCCCACAAGTGTCTC3' to examine expression of *PpGWD* genes. The linear amplification range was determined using *P. patens* actin primers[77] (Act sqRTFwd 5'AAGGCGAACAGGGAGAAGAT3'; Act sqRTRev 5'TCCACGAGACGACGTACAAC3'), and 20 cycles was chosen as the optimum number of cycles to use for all sqRT-PCR reactions.

GFP fusions and confocal microscopy. GWDa and GWDb cDNA were amplified by PCR using the following primers: PpGWD1a-GFP forward (5'ATGCAGAGACACGGAGTTCT3') and reverse (5'TTCAAACGAGACCCCAGATG3'); PpGWD1b-GFP forward (5'ATGAAGAGCTTCAGAGCTCA3') and reverse (5'TTCAAACAAGACCGCAAATG3') and cloned into pENTR/D-TOPO (ThermoFischer Scientific)). The inserts were transferred to the pMPL1382 GFP vector (http://labs.biology.ucsd.edu/estelle/moss2.html) using LR clonase. The GFP vectors were linearized using SphI and protoplasts were transformed with this DNA as described above. Protoplasts transiently expressing GFP were imaged using a Carl Zeiss LSM780 confocal microscope with an ELRYA S.1 super-resolution platform. GFP signal was detected using a laser excitation of 488 nm and GFP filter detection range of 490-579 nm. Chloroplast autofluorescence was detected using a laser excitation of 405 nm and chlorophyll filter detection range of 625-738 nm. Images were analysed with the Zen (black edition, version 2.3) imaging software (Carl Zeiss, Germany).

Growth and gametophore analysis. Plants grown on BCD medium or BCD supplemented with sugars were imaged and surface area determine by image analysis using ImageJ 1.52a[78]. Gametophore numbers were counted manually.

Starch, sugar and starch phosphate determination. Starch was measure enzymatically following digestion to glucose. Briefly, 20-50 mg plant material was placed in a microcentrifuge tube and sugars were removed by adding 1 mL 0f 80% (v/v) ethanol and heating at 95°C for 1 hour before the ethanol was decanted. This step was repeated twice more. After the final wash, 0.4mL of 0.2 M KOH was added and the sample heated at 95°C for one hour before the KOH was neutralized

through addition of 70 µL of 1 M acetic acid. At this point 1.5 mL of 100 mM NaAC (pH 5.5) was added alongside 65 U of amyloglucosidase (*Aspergillus niger*, Megazyme) and 60 U of thermostable α-amylase (*Bacillus* sp., Megazyme). This was incubated at 50°C for 1 hour before 100 µL was mixed with 200 µL of 100mM Tris-HCl (pH 7.0), 5mM $MgCl_2$, 1mM ATP and 1mM NAD. This was assayed at 340 nm in a microtitreplate reader for increase in absorbance after 1 U of hexokinase (Yeast, Megazyme) and 0.5 U of glucose 6-phosphate dehydrogenase (*Leuconostoc mesenteroides*, Megazyme). Glucose, fructose and sucrose were determined in the ethanol extracts by a previously described method[79]. Starch phosphate was determined by a previously published method[80] using starch purified[81] from protonemal tissue.

Statistical Analysis. Data were analysed by one-way analysis of variance followed by a BonferroniHolm post hoc test using Daniel's XL Toolbox add-in for Excel[82], version 7.3.2 (www.xltoolbox.net).

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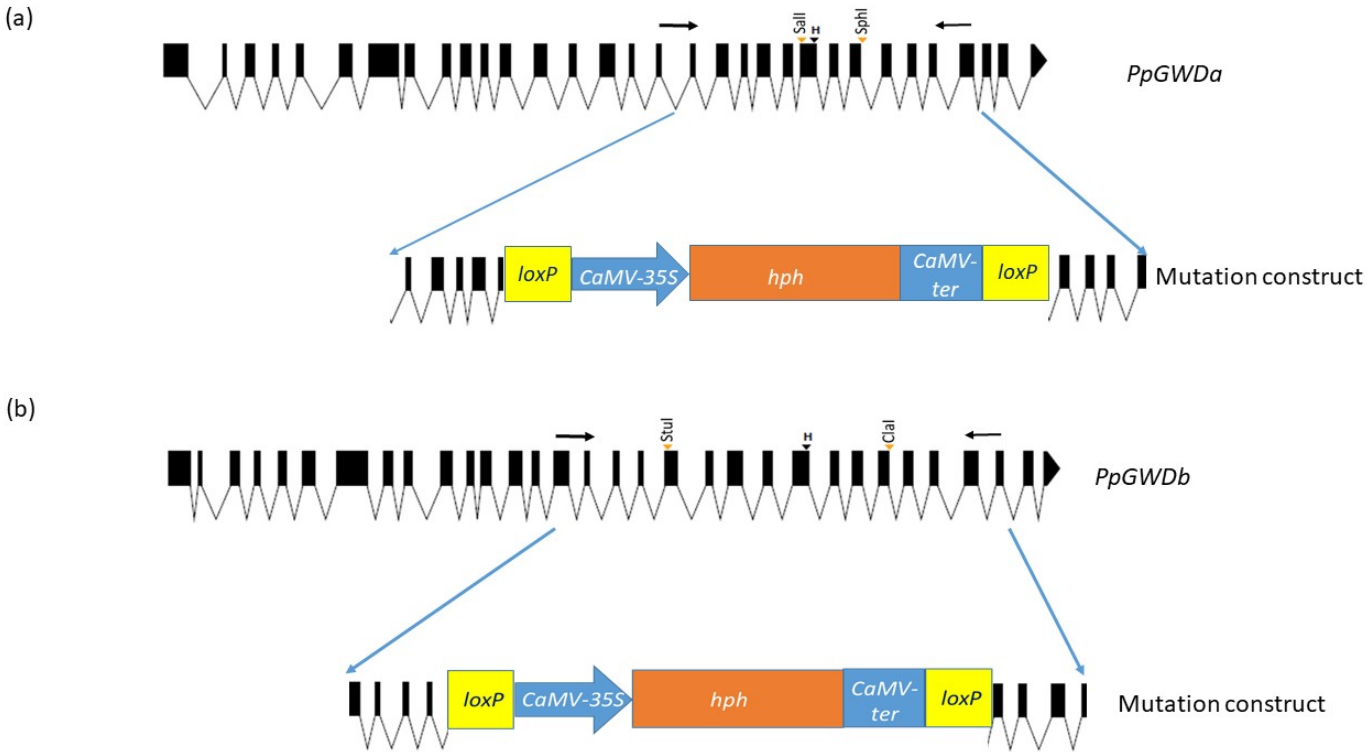
Author contributions

The project was conceptualized by J.R.L and J.K. Plants were initially transformed by N.T.M, J.F.J and S.M and all subsequent experimental work was performed by N.T.M. M.J.S helped produce the Cre recombiase expressing vector used to make the double mutants. The initial phylogeny

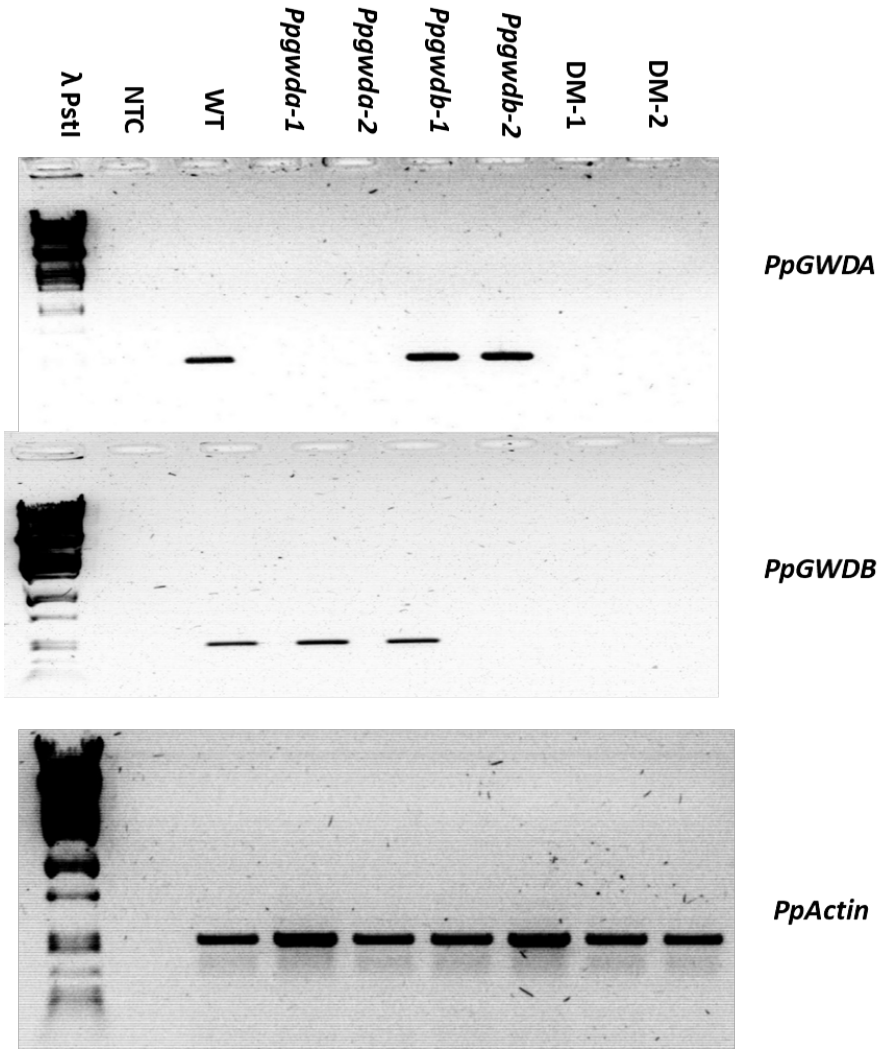
was established by E.P and expanded by J.R.L and K.O. The manuscript was written by N.T.M and J.R.L. All authors proofread the paper and provided feedback.

Competing interests

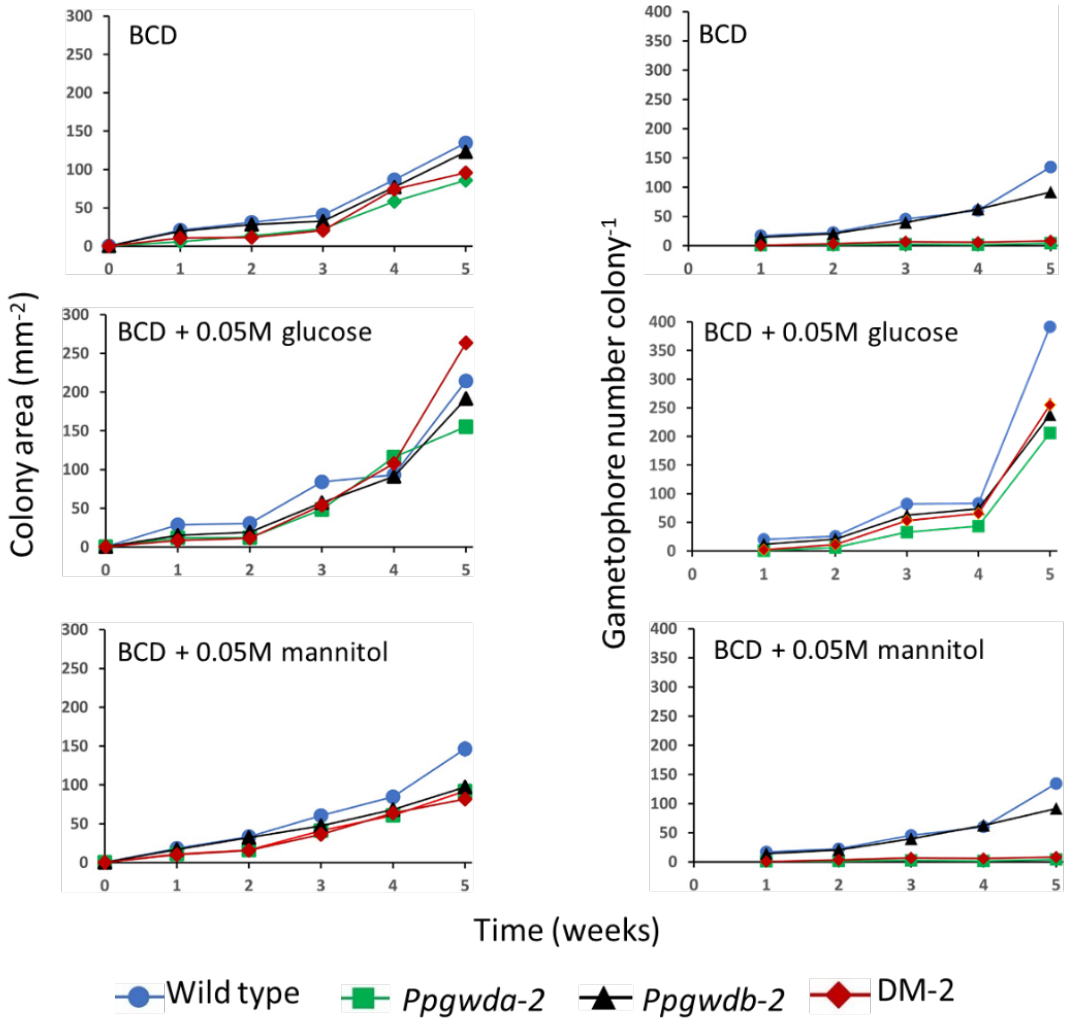
The authors declare no competing interests



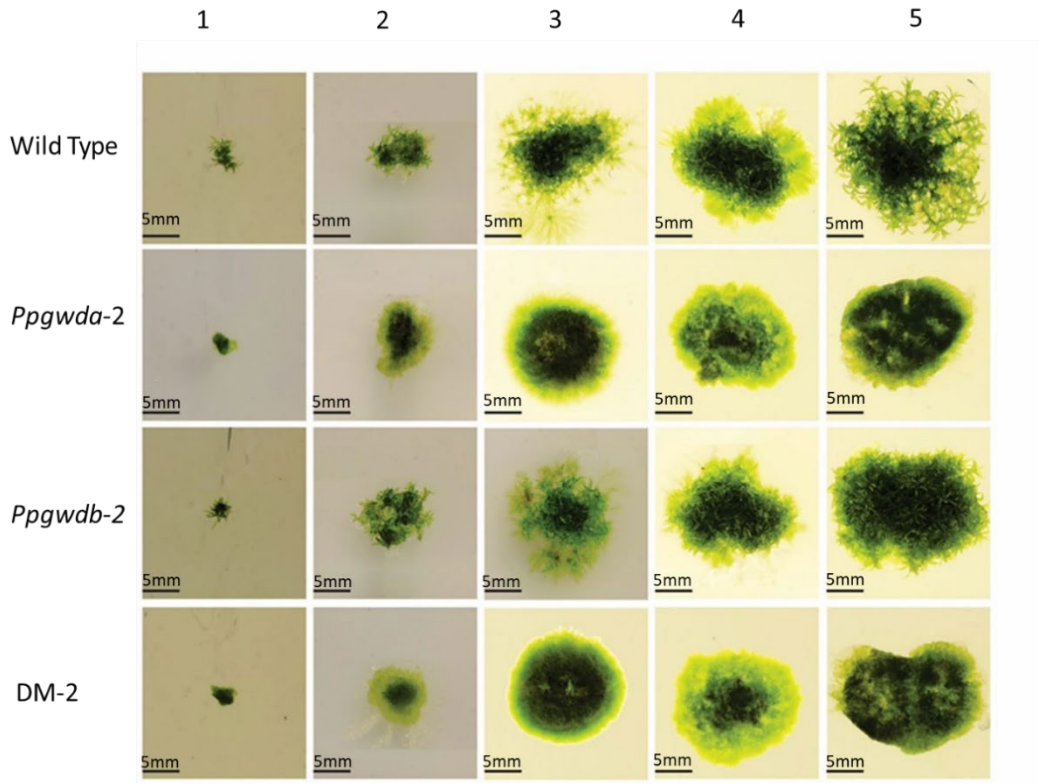
Supplementary Fig. 2.1. Construct synthesis for mutant production. PCR was used to amplify parts of *PpGWDa* (a) or *PpGWDb* (b) genomic DNA. Arrows indicate approximate primer binding sites within the genes and the H represents the position of the codon encoding the catalytically essential histidine. The amplicons were ligated into pJET1.2/blunt and digested with the restriction sites shown in the gDNA. A resistance cassette containing the cauliflower mosaic virus 35S promoter (*CaMV-35S*), *Klebsiella* hygromycin B phosphotransferase (*hph*) and the cauliflower mosaic virus terminator (*CaMV-ter*) flanked with *loxP* sequences were ligated between those sites.



Supplementary Fig. 2.2. Original DNA gels examining expression of *PpGWD1a*, *PpGWD1b* or *PpActin* via semi-quantitative RT-PCR in the wild-type (WT) or mutant lines. NTC denotes no template control and λPstI is DNA isolated form λ phage digested with PstI and used as a molecular weight marker.



Supplementary Fig. 2.3. Growth and gametophore number in the second set of mutant lines. Colonies were established on BCD media, BCD + 0.05M glucose and BCD + 0.05M mannitol and allowed to grow for 5 weeks. Data represents means of at least 3 colonies. Error bars are SEM and, if not visible, are within the symbol.



Supplementary Fig. 2.4. Colony morphology of wild-type and β mutant lines grown on BCD medium for 1, 2, 3, 4 and 5 weeks. Scale bar = 5mm.

Chapter Three

Research Article:

Knockout mutations of either ISA1 or ISA2 lead to water-soluble polysaccharide accumulation in *Physcomitrella patens*

Ms Mdodana was responsible for all experiments and analysis in this chapter, except for the phylogenetic tree.

Knockout mutations of either ISA1 or ISA2 lead to water-soluble polysaccharide accumulation in *Physcomitrella patens*

Abstract

Starch is the main source of carbon in plants and an important natural resource for feed, food and industrial raw materials. However, the details of the enzymes involved in this process in non-vascular plants are poorly understood. To learn more about this, we have studied two isoamylase type starch debranching enzymes (PpISA1 and PpISA2) in the moss *Physcomitrella patens*. Phylogenetic analysis showed *PpISA1* and *PpISA2* appear in separate clades alongside genes encoding type 1 or 2 enzymes from other plant species. Mutations in either *PpISA1* and/or *PpISA2* resulted in loss of isoamylase activity in native PAGE analysis, an accumulation of water-soluble polysaccharide (WSP) and reduced starch amounts. Analysis of recombinant PpISA1 and PpISA2 indicated that both encode active polypeptides. The WSP fraction of all the mutant lines contained significant amounts of glucose 6-phosphate, which was increased in the *Ppisa1* and double mutant lines compared to the wild type and *Ppisa2* lines. Furthermore, the mutant lines exhibited slow growth and disturbed gametophore development.

Background

Starch is a plant polyglucan that accumulates in plastids where it is stored as granules. Understanding its biosynthesis is important for several reasons. For example, some crops produce starch granules, which can be isolated for use in industrial processes. Altering starch polymer structure to make it more useful for industry has been an interest of biotechnologists for many years (Sonnewald and Kossmann, 2013; Zeeman et al., 2010). In addition, manipulation of starch synthesis and degradation in leaves has been shown to affect plant growth and productivity (Lloyd and Kossmann, 2019; MacNeill et al., 2017; Stitt and Zeeman, 2012). It is thought that its presence in photosynthetic tissue acts as a carbon store to buffer the plant during darkness and that an inability to synthesise enough starch, or to completely degrade it, leads to night time carbon starvation (Arias et al., 2014).

The pathway of starch synthesis occurs within plastids of most plants and appears relatively straightforward. In the first step of polymer formation starch synthases utilise ADP-glucose to form linear α1,4 linked glucans, after which starch branching enzymes introduce α1,6 linked branchpoints (Ball and Morell, 2003; MacNeill et al., 2017; Pfister and Zeeman, 2016; Sonnewald and Kossmann, 2013; Zeeman et al., 2010). In principle, these two activities catalyse all the reaction necessary to synthesise the two polymers that make up a starch granule, amylose (which is composed of mainly α1,4 linked chains) and amylopectin (which contains many short α1,4 chains linked together by α1,6 branchpoints). It has been known for many years, however, that amylopectin synthesis requires another class of enzymes, isoamylases (ISA; Ball et al., 1996; Zeeman et al., 1998). Their importance in this process has been demonstrated in transgenic and mutant plants lacking specific ISA isoforms (Ball and Morell, 2003; Pfister and Zeeman, 2016) as well as in yeast where the starch pathway was recently reconstructed (Pfister et al., 2016). These debranching enzymes cleave α1,6 branchpoints and mutations in some ISA isoforms leads to the accumulation of a branched water soluble polysaccharide (WSP), often known as phytoglycogen (Ball et al., 1996; Zeeman et al., 1998). Like amylopectin, this WSP contains α1,4 polyglucan chains that are linked together by α1,6 branchpoints, but it is more highly branched. This structural alteration can differentiate it from the starch polymer as it means that phytoglycogen, unlike starch, is water-soluble.

All plants contain multiple ISA isoforms that can be divided into three families, ISA1, ISA2 and ISA3 (Nougué et al. 2014; Qu et al 2018). Although both

ISA1 and ISA3 encode active polypeptides, in vascular plants ISA2 is catalytically inactive (Hussain et al., 2003; Sundberg et al., 2013).The relative importance of ISA1 and ISA2 in amylopectin synthesis depends on the plant species. In dicotyledonous plants, they form an enzyme complex where both subunits are required; else, the other becomes rapidly degraded. Mutations in either gene, therefore, lead to elimination of both ISA1 and ISA2 polypeptides and phytoglycogen accumulation (Bustos et al., 2004; Delatte et al., 2006; Sundberg et al., 2013). The situation in monocotyledonous plants is somewhat different as, although ISA1 and ISA2 form a heterocomplex (Kubo et al., 2010; Utsumi and Nakamura, 2006), ISA1 can also form a stable homodimer(Kubo et al., 2010; Lin et al., 2013). In the endosperm of these plants mutations in ISA1, but not ISA2, lead to phytoglycogen accumulation (Kubo et al., 2010). Their roles in non-vascular plants has only been examined in *Chlamydomonas reinhardtii* where ISA1, as in monocotyledonous plants, forms both a heterocomplex with ISA2 as well as a stable homocomplex (Sim et al., 2014). Unlike monocotyledonous plants, however, mutations in either ISA1 or ISA2 lead to phytoglycogen accumulation (Dauvillee, 2001a, 2001b; Mouille et al., 1989; Sim et al., 2014).

Given the alteration in ISA complex formation in different plant species over evolutionary time, it is of interest to examine other plants that are considered an intermediate species. One of these is the bryophyte *Physcomitrella patens* which last shared a common ancestor with both green algae and vascular plants approximately 450 million years ago (Rensing et al., 2008). There are several advantages in using *P. patens* in genetic studies, mainly due to the ease that knockout mutants can be produced using homologous recombination(Cove et al., 2006). Recently we have started examining starch metabolism in this plant through mutations of glucan water dikinase enzymes. This led to a starch excess phenotype and the formation of *P. patens* colonies lacking leafy shoot like gametophore tissue (Mdodana et al., 2019). In the current study, we examine starch biosynthesis through removing ISA1 and/or ISA2 activity in *P. patens*. We show that mutation in either enzyme lead to WSP accumulation and an alteration in gametophore development.

Methods

Plant material, growth, and sampling

The wildtype *P. patens* (Gransden) and mutant lines were grown as previously described (Mdodana et al., 2019), in sterileBCDAT media(1mM MgSO4, 1.85 mM KH_2PO4, 10 mM KNO_3, 45 mM $FeSO_4$, 1 mM $CaCl_2$, 9.93 µM H_3BO_3, 0.103 µM Na_2MoO_4, 0.266 µM $CoCl_2$, 0.191 µM $ZnSO_4$, 1.97 µM $MnCl_2$, 0.169 µM KI, 0.22 µM $CuSO_4$ and 0.57 µM $Al_2(SO_4)_3$, 5mM ammonium tartrate) at23±2°C with a photon flux of 50 µmol photons m^{-2} s^{-1} (Osram L 58V/740, Germany) and a 16 hours light/ dark regime.

RNA Isolation and cDNA preparation

Total RNA was extracted from *P. patens* tissue using Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, CA, www.qiagen.com), according to the manufacturer's instructions. RNA quantity was determined spectrophotometrically and through visualisation following separation on a 1% (w/v) agarose gel was used to assess the integrity and size range of the purified RNA. Synthesis of cDNA was performed with the purified RNA by using the RevertAidH Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA).

Construct preparation

Gene disruption cassettes were prepared as follows. Genomic DNA isolated from *P. patens* using the method of (Edwards et al., 1991) was used as template in PCR reactions utilising Q5 DNA polymerase (New England Biolabs) to isolate fragments of *PpISA1* (4040 bp) and *PpISA2* (5100 bp) genes (ISA1 FWD 5'GGCCACCTGTGAATGATGGA3' and ISA1 REV 5'CACCTACTAGCGCGTGTCAT3'; ISA2 FWD 5'GACGACGACATCATGCTTGC3' and ISA2 REV 5'GTCCCTCGACCATCTGCAAA3'). Amplicons were ligated into pJET1.2 (Thermo Scientific) before being digested with BamHI, releasing a fragment of *P. patens* gDNA from each construct. The BamHI fragment containing a Lox flanked gene disruption cassette, from pMBLH8a (Knight et al., 2002) was isolated through restriction digests, separation on 1% (w/v) TBE gels and subsequent gel purification (Wizard SV gel purification, Promega). The disruption cassette was ligated into the BamHI digested vectors containing either the *PpISA1* or *pPISA2* fragments using T4 DNA ligase (Thermo Scientific).

Protein expression cassettes were manufactured by amplifying *PpISA1* and *PpISA2*cDNA's from reverse transcribed *P. patens* RNA. PCR primers (ISA1 Forward 5'CACCATGGCGATGGCCATGG3'and ISA1

Reverse5'TGTTCTGAAAGATGAAGATGCATTCTAA3'; ISA2Forward 5'CACCATGAAAGACATAGCTATGGGG3' and ISA2 Reverse 5'TGGTGCGGGGCCTCAGTGA3') were designed to amplify cDNA lacking putative transit peptides, which had been determined based on analysis by ChloroP 1.1 (http://www.cbs.dtu.dk/services/ChloroP/). Amplicons were cloned into pENTR D-TOPO before being recombined into pDEST17 using LR Clonase (Life Technologies).

Production of mutants

pJET1.2 containing disruption cassettes flanked by *PpISA1* or *PpISA2* gene sequences were linearized through restriction with either NotI (*PpISA1*) or XbaI (*PpISA2*). Approximately 60 µg of DNA from either linearized construct was used to transform*P. patens*protoplasts using PEG mediated DNA uptakeaccording a previously published method (Cove et al., 2009). Protoplasts were regenerated on BCDATmedia, before undergoing two rounds of selection on hygromycin containing media, interrupted by a growth on non-selectionmedia to allow for elimination of colonies transiently expressing the hygromycin resistance gene.

Double mutants were produced following elimination of the disruption cassette from *Ppisa1* single mutant using Cre-recombinase, followed by transforming these lines with PpISA2-HygR-KO, according to the method described in Mdodana et al. (2019).

Examination of gene expression

Semi-quantitative RT-PCR was used to examine gene expression as previously described (Mdodana et al., 2019), using the following primers: ISA1sqFwd5'AATAGTTGGAATTGTGGAGAGG3' ISA1sqRev 5'CTTGGAAGTGTCTACCAAAGG3', ISA2sqFwd5'TTTCAATGGGAGGCAATGG3' ISA2sqRev5'GAGCCTCCAGGATCACC3'. The linear range of amplification was determined with the use of *P. patens* Actin primers (ActsqFwd 5'AAGGCGAACAGGGAGAAGAT3'; ActsqRev5'TCCACGAGACGACGTACAAC3', Aoki et al., 2004), and was established to be between 15 and 20 cycles. Thereafter 20 cycles were used for all sqRT-PCR reactions.

Protein extraction and quantification

Approximately 100 mg fresh protonemal tissue was homogenized in 250 µL ice cold protein extraction buffer (50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 5 mM DTT, 0.1% (v/v) Triton X-100, 0.01% (v/v) β-Mercaptoethanol). The extracts were clarified by centrifugation at 18,000 g for 20 min at 4°C. Supernatants were removed and soluble

protein concentrations determined using the Biorad Protein Assay Dye Reagent (Bradford, 1976) and bovine serum albumin (BSA; Calbiochem, San Diego, USA) as standard.

Zymogram analysis

Isoamylase activities were analysed using techniques modified from Bustos et al., 2004, Zeeman et al., 1998 and Zhu et al., 1998. Thirty micrograms of soluble protein from the crude protonemal extracts were loaded per well in a 7.5% (v/v) continuous native Tris-glycine (Sambrook and Russell, 2001) polyacrylamide gels lacking sodium dodecyl sulfate, but containing 0.1% (w/v) amylopectin from potato starch (Sigma-Aldrich®). Separation took place under non-denaturing conditions at 4°C and 200V for 3 hours. Gels were incubated at RT (20-25°C) in activity buffer (100 mM MES-KOH, pH 6.0, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM DTT and 0.1 mg/mL Acarbose) for 5-6 hours. Starch hydrolysing activities were revealed by staining the gels with Lugol's solution (40 mM KI, 13 mM I_2).

Recombinant protein construct preparation

For recombinant protein expression, ISA1 and ISA2 coding sequences (without their transit peptides) were amplified from *P. patens* cDNA by PCR using primers described in Appendix A. Amplicons were cloned into the Gateway® entry vector pENTR™ D-TOPO® (Invitrogen, Life Technologies®, USA-CA) according to the manufacturer's specifications. The cDNA's were recombined in frame with a His-tag in the protein-expression vector pMDC17using the Gateway® LR Clonase® Enzyme mix (Life Technologies®) as described in the manufacturer's instructions.

Recombinant Protein expression

Expression plasmids were transformed into either the *E. coli* BL21 codon plus RIPL strains (Agilent Technologies Company) or Rosetta BL21 (DE3) strains. Cultures containing 5 mL of the protein expression plasmid were grown overnight, in liquid LB medium at 37°C with shaking at 200 rpm. The overnight culture was then transferred to 200 mL LB and grown at 37 °C with shaking at 200 rpm. Isopropyl-D-

1-thiogalactopyranoside (IPTG) was added to a final concentration of 1mM as soon as the cells had reached an OD_{600} of 0.5. The induced cultures were then grown for 4 hrs at 20 °C, with shaking. Cells were then recovered by centrifugation (5000 g, 15 min at 4 °C) and the pellet resuspended in 20mL of lysis buffer (25 mM Tris-HCl, pH 7.5, 2mM EDTA). The cell suspension was sonicated on ice (Ten times 20 s bursts with 10 s cooling interval) to disrupt the cells and the resultant mixture was centrifuged (10 000 g for 5 min at 4 °C). Supernatant was collected and protein concentration quantified (Bio-Rad; Bradford, 1976).

Purification of the Histidine-tagged fusion protein

Crude protein extracts containing recombinant protein were transferred into a clean tube and further clarified by filtering through a 0.45 µm cellulose acetate membrane. Purification of the Histidine-tagged fusion protein was achieved by means of immobilized metal ion affinity chromatography (IMAC) using Ni-TED 1000 Packed columns (Protino®) according to the manufacturer's specifications. The supernatant was passed through the columns and washed with 10 volumes of column washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) at a flow rate of 1 mL/min. After several rounds of washing, the protein of interest was eventually eluted from the column using the elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM Imidazole) in three separate 1.5 mL fractions. SDS-PAGE was used to determine protein purity and amount in the different fractions.

SDS-PAGE

Samples were heated in Laemmli buffer (375 mM Tris-HCl, pH6.8, 9% (v/v) SDS, 50% (v/v) Glycerol, 0.03% (w/v) Bromophenol blue) at 100 °C for 5 – 10 min. Denatured samples were loaded in 10% (w/v) sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and separated at 120 V for 2 hrs at room temperature (Sambrook and Russell, 2001). The gels were then stained overnight with Comassie brilliant blue (0.1 % (w/v), 10% (v/v) acetic acid, 20% (v/v) HPLC grade methanol) before subsequent decolourization by incubation 10% (v/v) acetic acid, 50% (v/v) methanol.

Determination of starch and water-soluble polysaccharide

Starch and WSP amounts were determined enzymatically after perchloric acid extraction from *P. patens* tissue using a previously published method (Facon et al., 2013).

Phylogenetic analysis

A tBLASTn search was performed using the *Arabidopsis thaliana* ISA1 (*AtISA1*) protein sequence as query against the *Physcomitrella patens* genome on Phytozome (V1.6, http://www.phytozome.net) to identify putative isoamylase encoding genes. Isoamylase DNA sequences were also collected either from NCBI or from Phytozome for 11 representative land plants (Vitis *vinifera, Helianthus annus*, Zea *mays, Sorghum bicolor, Aegilops tauschii, Arabidopsis thaliana, Arabidopsis lyrata, Olea europaea, Prunus persica, Solanum tuberosum, Physcomitrella patens)* and *Escherichia coli*. DNA sequence alignment was performed with clustalW package and submitted to MEGA X (Kumaret al., 2018) for construction of a phylogenetic tree. The neighbour-joining method(Saitou and Nei, 1987) with 1000 replications in the bootstrap test was used to assess the statistical reliability of the tree topology (Felsenstein, 1985). The evolutionary distances were computed using the Tajima-Nei method (Tajima and Nei, 1984) and are in the units of the number of base substitutions per site. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). This analysis involved 40 nucleotide sequences and the codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated, and fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 1289 positions in the final data set.

Growth analysis

Plants grown on BCD plates (ammonium tartrate-free BCDAT media) were photographed and surface area measured with image analysis, using the ImageJ program (Schneider et al., 2012). Leafy shoots were individually picked with tweezers from each plant and carefully laid down on 0.8 %(w/v) agarose for analysis. Measurement of gametophore length was also performed using ImageJ on images on photographs of the leafy shoots, with at least five colonies in which 30 to 60 gametophores were used to generate data for each moss plant line. Counting of the total number of gametophores was done manually.

Statistical Analysis

Data were analysed by one way analysis of variance followed by a Bonferroni-Holm post hoc test using Daniel's XL Toolbox add-in for Excel[82], version 7.3.2 (www.xltoolbox.net).

Results

Physcomitrella patens contains single genes encoding ISA1, ISA2 and ISA3 isoforms

We used *Arabidopsis thaliana* ISA1 (NCBI accession NM_129551.3) protein sequence to search for the presence of ISA like genes within the *Physcomitrella* genome at Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#!search?show=BLAST) using the tBLASTn algorithm. This identified eight putative loci situated on chromosomes 1, 3, 5, 8, 13, 14, 16 and 27. Three of these (Pp3c14_11860V3.1, Pp3c8_12820V3.1, and Pp3c_14050V3.1) revealed a high degree of protein similarity (50-70 % identity) to isoamylases from *Arabidopsis*, while the others (Pp3c1_1810V3.1, Pp3c5_8810V3.1, Pp3c16_13360V3.1, Pp3c27_2570V3.1, and Pp3c13_17460V3.1) only demonstrated between 24-30 % identity. This indicates that *Physcomitrella* contains three isoamylase type enzymes and five genes encoding other glucosyltransferases. BLASTP searches against the *Arabidopsis* genome using predicted protein sequences of the *Physcomitrella* genes indicates that one (Pp3c13_17460V3.1) of the five is most similar to limit dextrin enzyme (LDA; 58% identity to the *Arabidopsis* protein), while the other four (Pp3c1_1810V3.1, Pp3c5_8810V3.1, Pp3c16_13360V3.1, Pp3c27_2570V3.1) show similarity to starch branching enzymes (between 41- 61% identity to the *Arabidopsis* proteins).

We produced a phylogenetic tree (Fig. 3.1) using the putative isoamylase orthologues to examine the relationships of the three sequences predicted for the *Physcomitrella* genome with other isoamylase genes from a variety of species, including green algae, red algae, vascular and non-vascular plants. As expected, (Nougué et al., 2014; Qu et al. 2018), this demonstrated that the isoamylase genes fall into three different clades, ISA1, ISA2 and ISA3. All three *P. patens* isoamylases group together with isoamylases from vascular plants with the Pp3c14_11860V3.1 grouping in the ISA1 clade, Pp3c8_12820V3.1 in the ISA2 clade and Pp3c3_14050V3.1 in the ISA3 clade. We will refer to these respectively as *PpISA1*, *PpISA2* and *PpISA3*.

Next, we compared the amino acid sequences of the two *Physcomitrella* proteins with a range of ISA1 and ISA2 polypeptides from other plants. PpISA1 and PpISA2 contain the four well-known consensus regions (motifs I-IV) conserved within the glycoside hydrolase family 13 of starch hydrolytic enzymes (Takata et al., 1992; Jesperson et al., 1993; Svensson et al., 1994; Nakamura et al., 1996; MacGregor et al., 2001; Fig. 3.2). In addition, two conserved motifs, V and VI, that are thought to occur in all plant debranching enzymes (Beaty et al., 1999) were also found in both

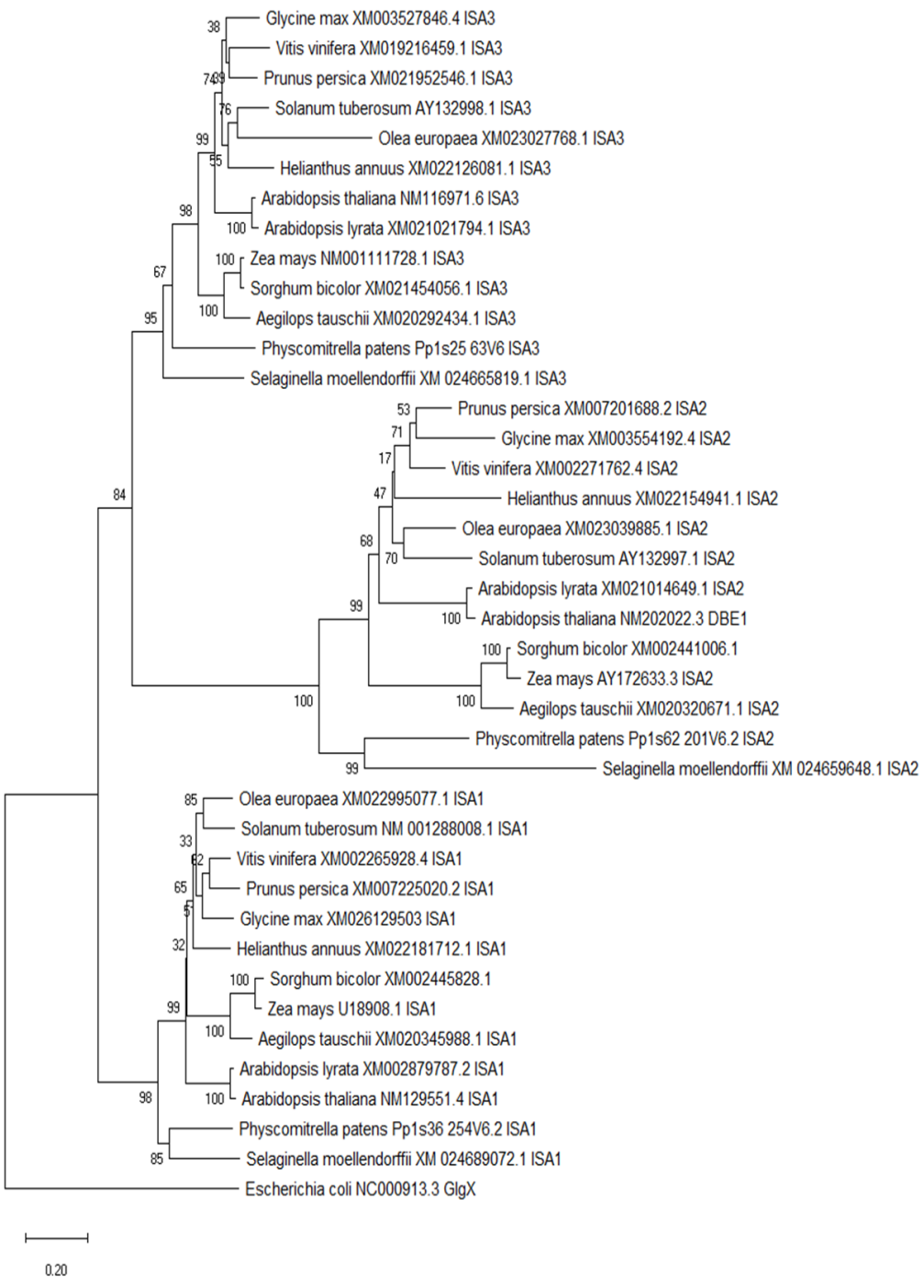


Figure 3.1. Phylogenetic analysis of ISA isoforms from various plant species. The tree was computed from the aligned sequences using the neighbour-joining method. The numbers above the branches are bootstrap values signifying support The *E. coli* GlgX Sequence was used as an outgroup.

PpISA1 and PpISA2. The four original conserved sequence regions are involved in the formation of the active site and contain the catalytic and substrate binding residues, whereas the two additional conserved sequence regions may contain residues connected to the enzymes specificity (Janeček, 2002). Eight amino acid residues that are highly conserved in all members of this family (Asp-377, Val-379, His-382, Arg-450, Asp-452, Glu-527, His-619, Asp-620; Hussain et al., 2003) were also found (Fig. 3.2). According to Macgregor et al (1993), three of these residues, Asp-452, Glu-527 and Asp-620 are essential for catalytic activity. While all eight of these residues are present in PpISA1, only three (Asp-377, Val-379 and His-382) are conserved in PpISA2 (Fig. 3.2). The other five residues in PpISA2 are altered as follows: R450C, D452K, E527D, H619P and D620Y. In ISA2 isoforms from other species, the replacement of His619 with Asn was conserved in maize, *Arabidopsis*, potato and rice while it was replaced with Leu in *Chlamydomonas*. Asp-620 was changed to glycine *C. reinhardtii* while in maize, *Arabidopsis*, potato and rice this amino acid was replaced by serine.

Both *PpISA1* and *PpISA2* encode active isoamylases

To examine the activities of PpISA1 and PpISA2, we amplified cDNA's lacking the putative transit peptides and manufactured constructs where these sequences were fused in frame with a histidine tag. After expression in *E. coli*, recombinant protein was purified using a nickel column. Calculated molecular weights of the fusion proteins are 91.35 kDa for PpISA1 and 104.67 kDa for PpISA2 (Fig. 3.3c; Supplemental Figur 3).Staining of SDS-PAGE gels demonstrated that both ISA1 and ISA2 polypeptides had been purified to apparent homogeneity and that the molecular weight of each protein approximated their calculated masses.

Approximately 20 µg of each protein were separated via native-PAGE on a gel impregnated with 0.1% (w/v) amylopectin. Following overnight incubation, the gel was stained with iodine and PpISA1 yielded at least three clearly discernible activity bands indicating the formation of several homocomplexes. Interestingly, PpISA2 also showed activity with three activity bands appearing, which were present in approximately the same abundance as ISA 1 (Fig. 3.3d).

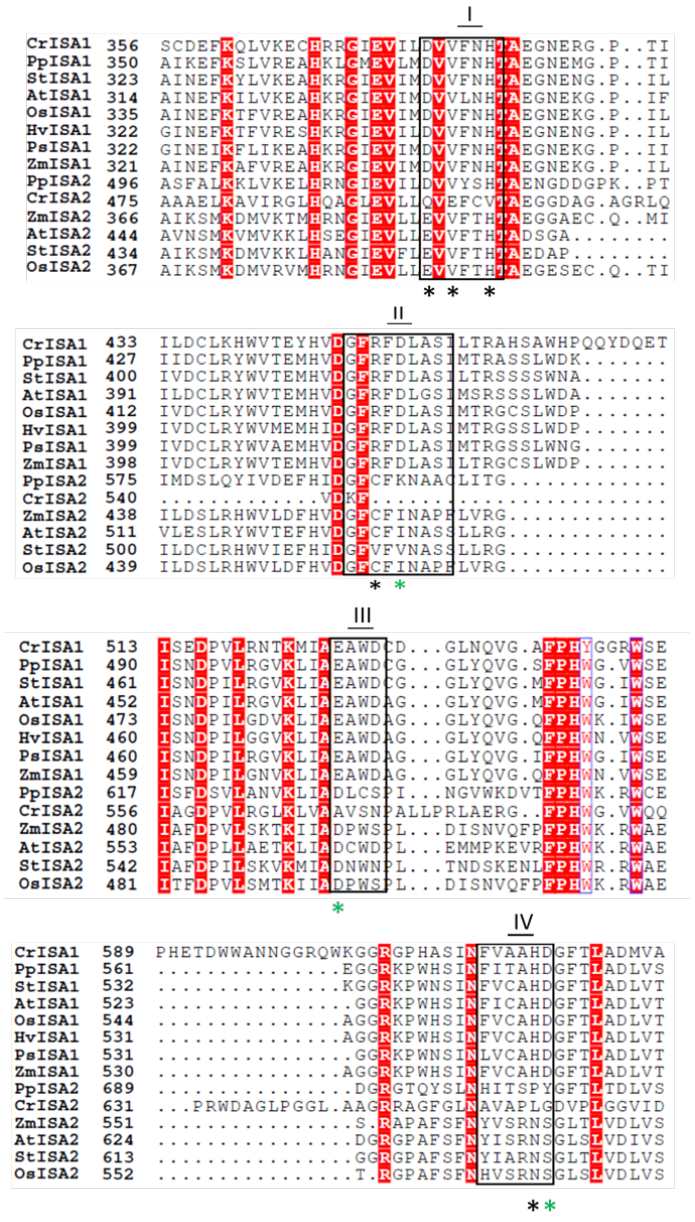


Figure 3.2. Multiple sequence alignment of ISA isoforms. The protein sequences of the type 1 and type 2 ISA isoforms were aligned with ISA sequences from *Chlamydomonas*, Potato, *Arabidopsis*, rice, wheat, pea and maize obtained from Phytozome and the NCBI. The alignment was performed using ClustalW and Espript. The four conserved motifs (i-iv) present in all the enzymes of the GH13 family are boxed. Asterisks mark the eight residues conserved in all active members of this family. Green asterisks indicate the catalytic triad residues known to be responsible for the catalytic activity. Letters in red boxes are amino acid residues that are strictly identical and blue framed columns indicate similar residues.

Insertions in *ISA* genes lead to elimination of transcript and loss of an activity band

To examine the roles of PpISA1 and PpISA2 we manufactured knockout mutants through homologous recombination (Supplemental Figure 1). We manufacture two *Ppisa1* mutants (*Ppisa1-1* and *Ppisa1-2*), two *Ppisa-2* mutants (*Ppisa2-1* and *Ppisa2-2*) and two double mutants (DM-1 and DM-2). Inserts within the genome were analysed by PCR using gDNA as template and two sets of primers (Fig. 3.3a). The first set bind either side of the homologous recombination site and were used to detect the presence of wild type alleles (amplicon sizes of 4040 bp for *PpISA1* and 5100 bp for *PpISA2*), and deletions within the *PpISA1* locus in double mutants (DM) where the amplicon size is approximately 500 bp. This confirmed the presence of the wild type *PpISA1* alleles within the *Ppisa2* mutants and the presence of wild type *PpISA2* within *Ppisa1* mutants. The second primer set consisted of one primer binding upstream of the insertion and another within the disruption cassette. This confirmed the presence of the cassette within either the *PpISA1* or *PpISA2* loci in both single and double mutants (Fig. 3.3a). Use of both primer sets demonstrates the presence of mutant alleles at both loci in the DM. Next, we examined gene expression using semi-quantitative RT-PCR and confirmed that RNA transcribed from either Ppc314_11860 or Pp3c8_12820 loci was abolished whenever the gene disruption cassette was present (Fig.3.3b; Suppleemntal Fgure 2).

To investigate whether mutations at the *PpISA1* and *PpISA2* loci resulted in loss of isoamylase activity, we loaded crude protein extracts from the protonemal tissue of the wild type and mutant lines onto amylopectin-containing gels. Proteins were separated under native conditions and the gels assessed for debranching enzyme activity after an overnight incubation step and iodine staining (Fig. 2.3d). Several activity bands were observed: Three of these appeared to lead to clearing of amylopectin from the gel, indicating removal of glucan (bands 1, 3 and 4). A fourth (band 2) stained brown indicating an increase in branch number within the amylopectin embedded in the gel. One of the degrading activities (band 1) was present in crude extracts of the wild type but lacking in all single and double mutant lines.

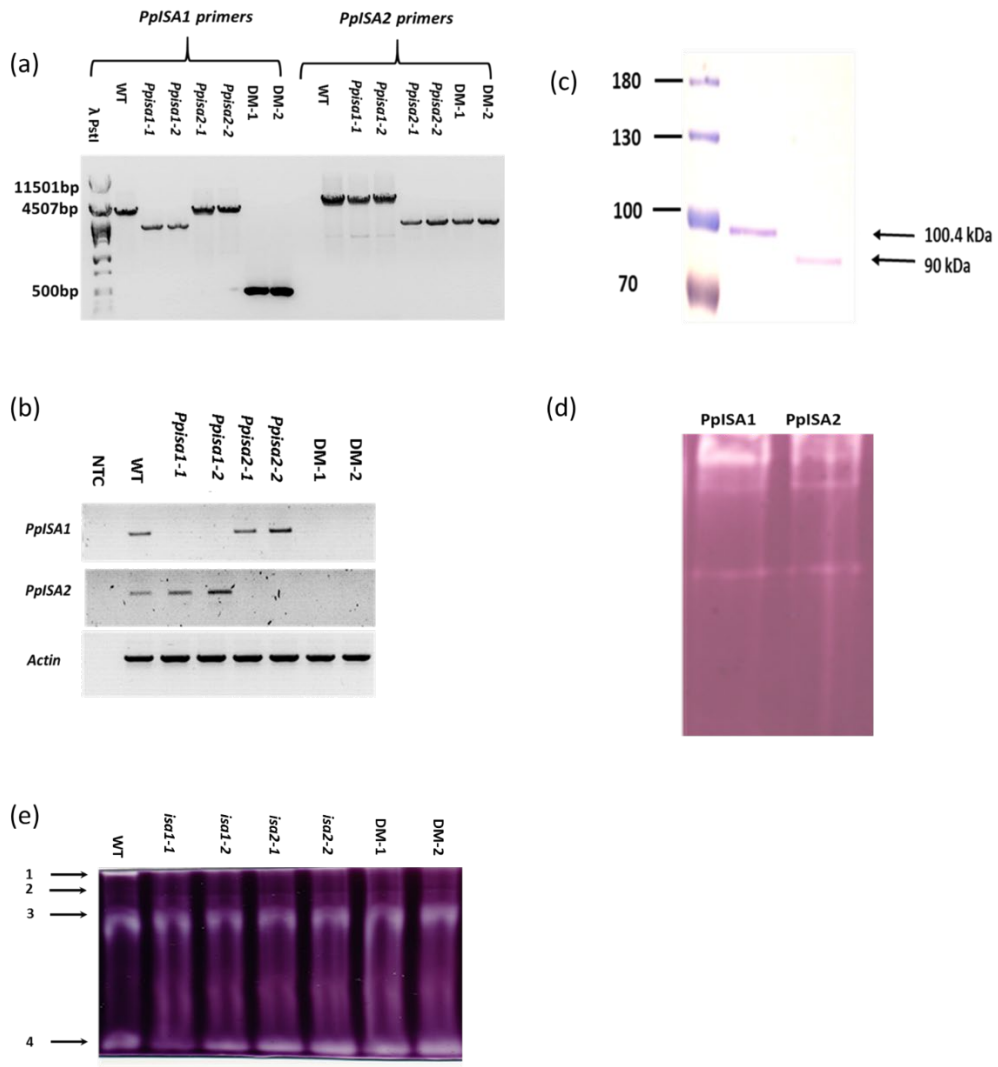


Figure 3.3. Examination of ISA in *P. patens*. (a) Verification of *Ppisa1*, *Ppisa2* and DM knockouts. PCR amplicons using gDNA as a template and either *PpISA1* or *PpISA2* specific primers. λ Pst1 is λ phage DNA digested with *PstI*. (b) Semi-quantitative RT-PCR analysis of *PpISA1*, *PpISA2* and *Actin* expression in the WT, *Ppisa1*, *Ppisa2* and DM lines. For the negative control (NTC), no template was added for each of the primer sets. Original gels are shown in supplementary data. (c) Coomassie brilliant blue stained SDS-PAGE gel of. His-Tagged purified recombinant PpISA1 and PpISA2 proteins. Lane 1 is PageRuler molecular weight marker. PpISA1 (90 KDa) and PpISA2 (100.4 KDa) are shown in lanes 2 and 3 respectively. (d) Purified recombinant PpISA1 and PpISA2 His-tagged protein samples were analysed for isoamylase activity on amylopectin-containing native gels (e) Activity gel detection of starch-modifying activities from *P. patens* crude protein extracts. Thirty micrograms of crude protein from protonemal tissue were separated on amylopectin containing gels. Iodine was used to visualise alterations in amylopectin amounts

WSP accumulates in the mutant lines and is phosphorylated in the *isa1* and double mutants

We conducted experiments to assess the amount of WSP and starch present in tissue of the plant at the end of the photoperiod. The wild type contained approximately 8 mg starch gFW^{-1} and this was reduced by about 90% in all the mutant lines (Fig. 3.4a). Conversely, a strong accumulation of WSP was found in all lines but was increased from approximately 2 mg gFW^{-1} in the WT to between 9 mg $gFW^{-1}$and 12 mg gFW^{-1} in the mutant lines (Fig. 3.4b).We examined the amount of covalently bound phosphate in the WSP. Within the WT and *Ppisa2* mutant lines, WSP phosphate content was approximately 1nmol G6P mg hexose $equivalents^{-1}$ and identified increased amounts (approximately 3nmol G6P mg hexose $equivalents^{-1}$) in WSP from both *Ppisa1* and double mutant lines (Fig. 3.4c).

Single and double ISA mutants demonstrate alterations in both growth and gametophore development

Analysis of both single and double mutant lines that were grown on BCD medium, demonstrated some slight but significant ($p<0.05$) differences in colony diameter compared with the wild type (Fig. 3.5a and 3.5c). We also found that the mutant lines developed gametophores approximately 2 weeks later than the wild type following the inoculation of 2mm diameter protonemata on BCD media. Although colonies from the mutant lines developed gametophores later, we noted that these were fewer in number compared to the wild type ($p<0.05$), which contained almost twice the number of gametophores (Fig. 3.5a and 3.5c). Furthermore, gametophores formed by the mutants were shorter in length than those from the wild type, with the DM showing the most severe effect (Fig. 3.5b and 3.5d).

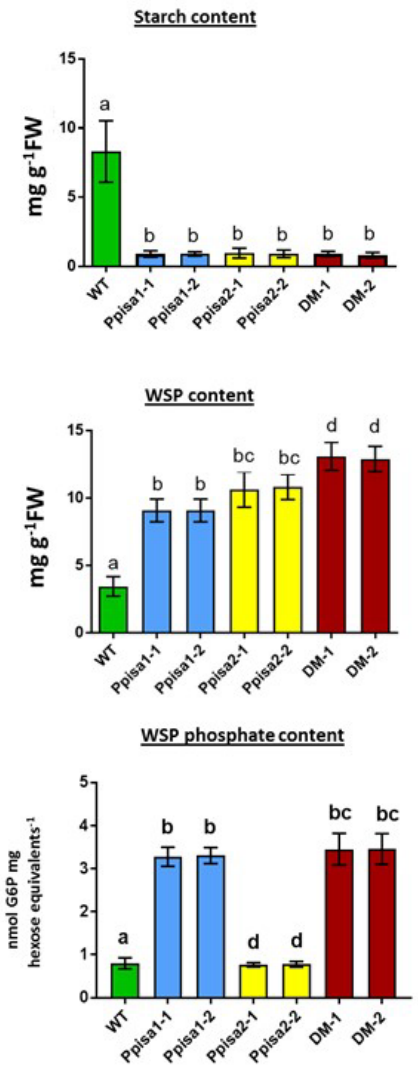


Figure 3.4. Carbohydrate analysis of *P. patens.* Colonies from the WT and mutant lines were harvested at the end of the photoperiod. Soluble and insoluble fractions were extracted using perchloric acid. (a) Starch and (b) phytoglycogen contents were determined following enzymatic hydrolysis to glucose. (c) Glucose 6-phosphate present within phytoglycogen. Values are means of five biological colonies and error bars are SEM. Significant differences between groups are indicated by different letters (One-way ANOVA and subsequent Bonferroni *post hoc* test, $p<0.05$).

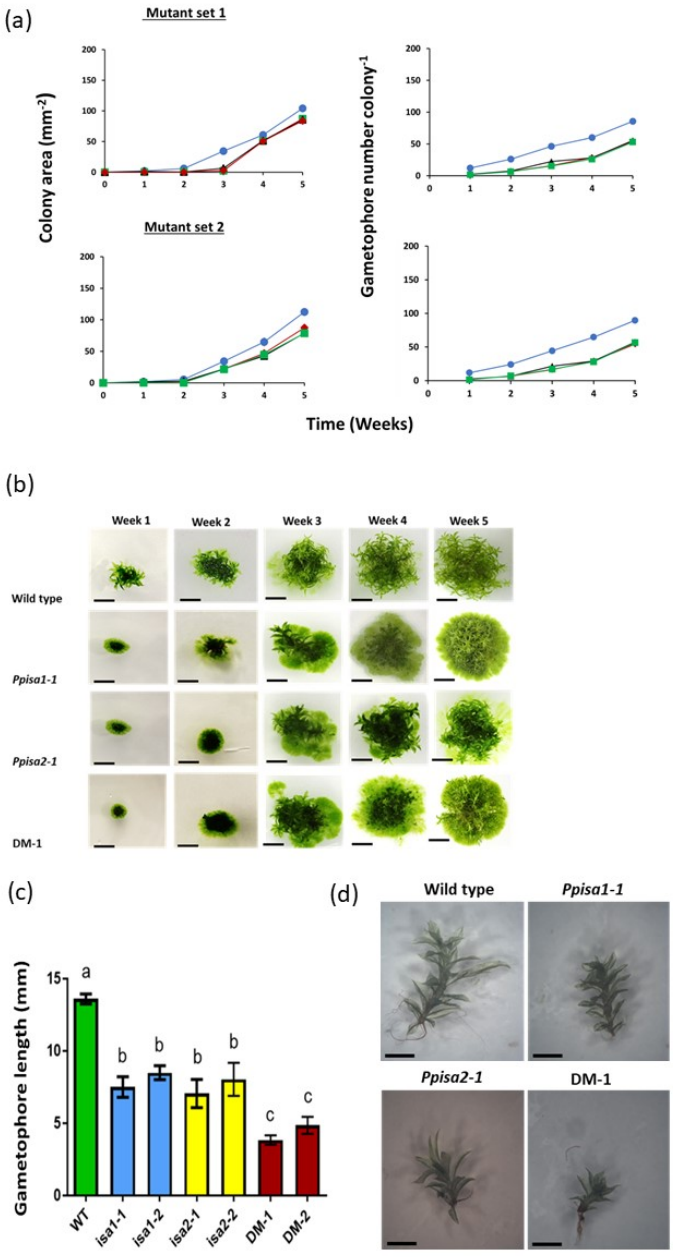


Figure 3.5. *Physcomitrella patens isa* mutants are affected in development. (a) Colony diameter and gametophore numbers after 5-week growth on BCD media. Wild type, *Ppisa1*, *Ppisa2*, DM. Data represents means ± SEM of at least 5 colonies. If not visible error bars are within the symbol. (b) Colony morphology of the WT and mutant lines allowed to grow for 5 weeks on BCD media. Scale bar = 5mm. (c-d) Gametophore length of the WT and mutant lines. Gametophores from each plate grown for 5 weeks were randomly selected and the lengths measured under a microscope. Different letters indicate significantly different results between genotypes based on one way ANOVA and Bonferroni *Post hoc* test ($p<0.05$). Values represent mean ±SEM , n= 30.

Discussion

Isoamylase-type debranching enzymes are known to play an important role in the biosynthesis and crystallization of starch. Many mutants have been isolated in vascular plants where elimination of either ISA1 or ISA2 lead to accumulation of WSP at the expense of that polyglucan. This process appears to be universal in plants, as similar results have been observed in both *Chlamydomonas reinhardtii* vascular plants and even a Cyanobacterium (Mouille et al., 1996; Zeeman et al., 1998; Dauvillé et al., 2001; Burton et al., 2002; Cenci et al., 2013; Delatte et al., 2005; Wattebled et al., 2005, Kobayashi et al., 2016). In this study, we wanted to broaden existing knowledge of this process by examining the functions of ISA1 and ISA2 in the non-vascular plant *P. patens*. Its evolutionary position within the plant lineage, sequenced genome together with the high efficiency for gene targeting, makes it an ideal model to study the roles of these enzymes.

To learn more about the function of isoamylases in these plants, we identified eight sequences within the *P. patens* genome encoding proteins that demonstrate significant similarity to *Arabidopsis* ISA1. Three of these are most closely related to isoamylases, while the others are more similar to other members of the α-amylase gene family, namely limit dextrinase and starch branching enzymes. Phylogenetic analysis indicates that the three isoamylase like gene group into previously identified *ISA1*, *ISA2* and *ISA3* clades (Fig. 3.1) and we named them, therefore *PpISA1*, *PpISA2* and *PpISA3*. As an initial attempt to examine the roles of these proteins in *P. patens* we decided to concentrate on the *ISA1* and *ISA2* like genes because they are known to be fundamentally important in formation of the starch polymer.

First, we decided to examine their catalytic activity through purification of recombinant protein. The activity of ISA1 and ISA2 isoforms has been studied in many vascular plants and in these species ISA1 is known to be active, while ISA2 is not (Fujita et al., 1999; Hussain et al., 2003; Utsumi and Nakamura, 2006; Facon et al., 2013; Sundberg et al., 2013; Streb and Zeeman, 2014).Indeed, ISA2 lacks many of the amino acid residues thought to be essential for catalysis (MacGregor et al., 2001; Hussain et al., 2003). In non-vascular plants, ISA1 from *C. reinhardtii* has been shown to be active (Sim et al., 2014), while the activity of CrISA2 has not been examined. The active site in PpISA1 contains all the amino acids thought to be necessary for catalysis (Fig.3.2), so it is unsurprising that purified recombinant PpISA1 demonstrates activity on a native gel (Fig. 3.3d). However, the activity

demonstrated by purified recombinant PpISA2 (Fig. 3.3d) was unexpected as the active site within this polypeptide contains substantial numbers of substitutions in amino acids thought to be important for activity (Fig. 3.2). In angiosperms, lack of ISA2 catalytic activity has been attributed to substitutions in some of the 8 amino acids present within the active site. Sequence analysis demonstrates that six of these residues are altered and only two are conserved (Val-379 and His-382) consequently resulting in loss of ISA2 activity in these plants. In PpISA2, one of the five amino acids known to be altered in angiosperms is unaltered, and we have unexpectedly observed activity in native gels. This could indicate that the unaltered amino acid (Asp-377) is essential for isoamylase activity. A less likely alternative is that the tertiary structure of PpISA2 allows activity irrespective of the amino acids present in the active site. Interestingly Asp-377 is also present in ISA2 from *Chlamydomonas*, so it would be interesting to examine ISA2 activity from this algae. We cannot however, rule out the final possibility that the activity comes from contamination of the *E. coli* GlgX debranching enzyme and this will need to be examined in more detail through purification of crude extracts from cells carrying the empty vector or through complementation of a *glgx* bacterial mutant with the *Physcomitrella* genes.

To examine the roles of ISA1 and ISA2 isoforms in *Physcomitrella*, we manufactured single (*Ppisa1* and *Ppisa2*) and double (DM) knockout mutants by homologous recombination (Fig. 3.3a). Mutant alleles led to elimination of mRNA accumulation of the mutated gene but did not affect transcript amount of the other isoamylase encoding gene (Fig. 3.3b). Examination of enzyme activity from crude extracts via native gels revealed at least four activity bands, demonstrating the presence of various starch hydrolytic enzymes (Fig. 3.3e). One band was absent in the extracts of all the single and double mutants (Fig. 3.3e). This most likely represents an ISA1/ISA2 heterocomplex, as has been found to be present in both green algae (Dauvillée et al., 2001a; 2001b; Sim et al., 2014) and vascular plants (Hussain et al., 2003; Bustos et al., 2004; Delatte et al., 2005, Wattebled et al., 2005; 2008; Facon et al., 2013; Kubo et al., 2010). The fact that the activity is lost in both *Ppisa1* and *Ppisa2* single mutants, and that no other activity appears, indicates that the complex is unstable when either protein partner is lost. This suggests that the situation in *P. patens* is more like dicotyledonous plants than *Chlamydomonas reinhardtii* or cereals, where loss of ISA2 can be compensated for by an ISA1 homocomplex.

Loss of isoamylase activity results in a dramatic decrease of the amounts of starch (Fig. 3.4a) accumulating alongside the appearance of increased amounts of WSP (Fig. 4b). This shows that PpISA1 and PpISA2 play a similar role in starch metabolism as in other plants. We examined covalently bound phosphate levels in the WSP of the plants that we produced and found significant amounts. In WT and *Ppisa2* mutants these were approximately 20% of the amount known to exist in *P. patens* starch (Mdodana et al., 2019), but this was significantly increased in WSP isolated from *Ppisa1* and DM lines (Fig. 3.4c). This indicates that WSP acts as a substrate for the glucan, water dikinase (GWD) which phosphorylates starch. The difference in phosphate levels in the WSP from the varying lines could be caused by alterations in structure and GWD is known to preferentially phosphorylate specific chain lengths (Mikkelsen et al. 2004). The significance that this plays on WSP metabolism is, however, unclear and needs to be examined through production of double mutants lacking both PpISA1 and PpGWD.

Colonies from all mutant plants exhibited significant differences in growth when grown on BCD medium, developing fewer gametophores which demonstrate shorter shoot length compared to the wild type (Fig. 3.5). These observations demonstrated that WSP accumulation at the expense of starch affects various aspects of the growth and development of *P. patens* gametophores. Defects in carbon metabolism in *P. patens* are known to affect gametophore development. For example, mutations of the major glucose phosphorylating hexokinase led to plants with increased gametophores when grown on media supplemented with glucose. (Olsson et al., 2003). On the other hand, mutation of glucan, water dikinase (*PpGWD)* eliminated gametophore development; a phenotype that could be chemically complemented through addition of glucose to the growth media (Mdodana et al., 2019; Chapter 3). That mutation, however, did not affect growth rates. This indicates that carbon pools within *P. patens* influence the initiation of gametophores, although a direct mechanism as to how this occurs is currently lacking. In this study, the mutant plants accumulate less starch and more WSP, so there could be an osmotic effect leading to the observed growth phenotype through some transcriptional or post-translational mechanism. Many pathways are known to affect gametophore development and the control over these is poorly understood. Clearly more work needs to be done to help in elucidating the mechanisms linking carbon metabolism to growth and gametophore development in *P. patens,* and the mutants produced in this chapter will help in providing material that can be used in this regard.

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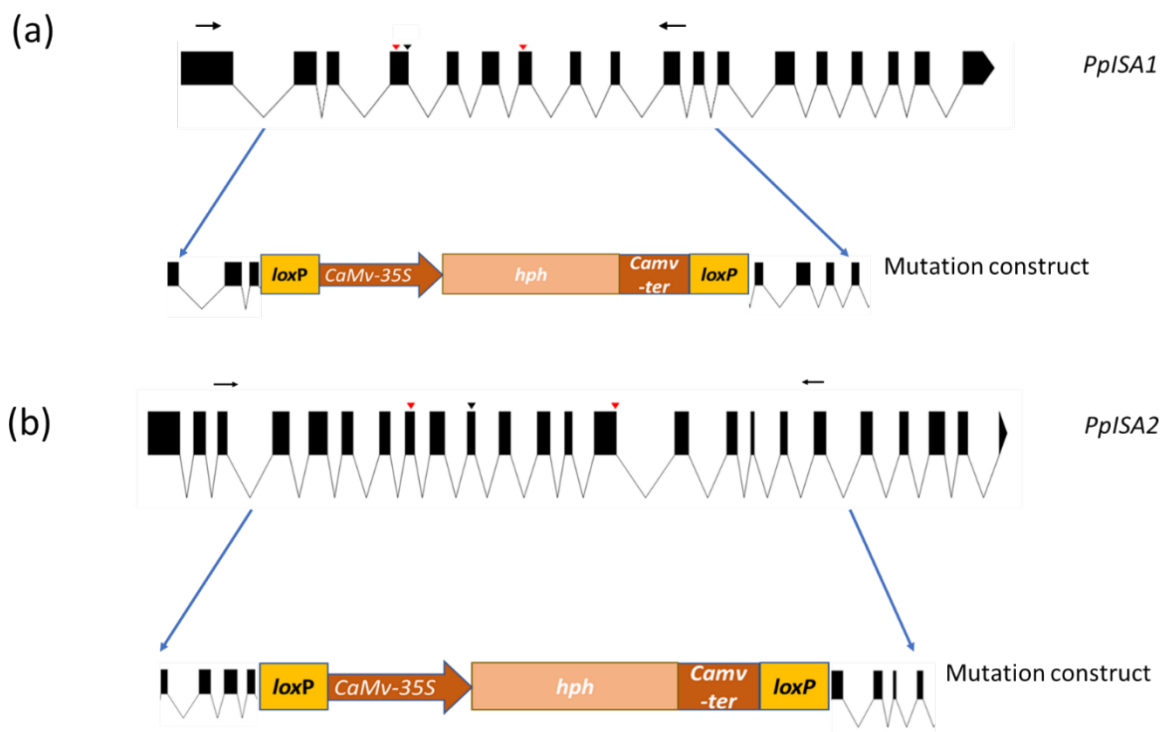
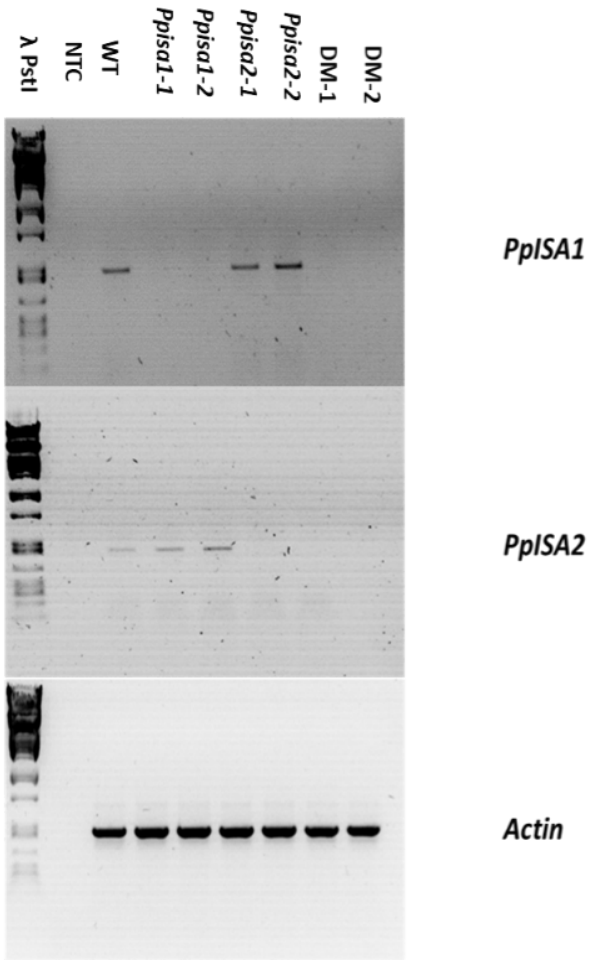
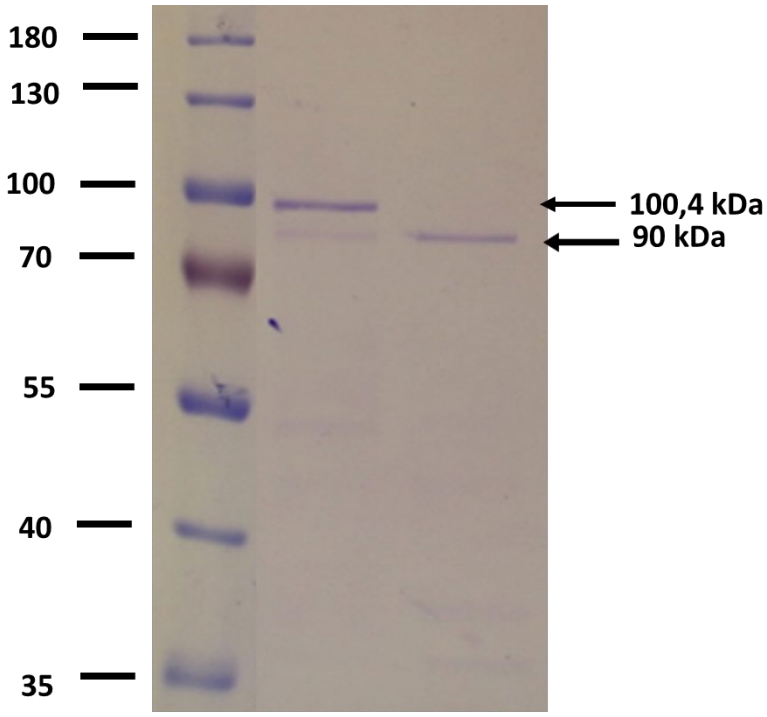


Supplemental Figure 3.1. Targeting construct generation for mutant production. PCR was used to amplify parts of *PpISA1* (a) or (b)*PpISA2*from genomic DNA and ligated into pJET1.2/blunt. The approximate primer binding sites within the genes are shown by black arrows. Red triangles indicate the active site and black triangles represent restriction sites and the area in which the codons were replaced by the *loxP* flanked *Klebsiella* Hygromycin B phosphotransferase resistance cassette (*hph*) which contains the cauliflower mosaic virus 35S promoter (*CaMV-35S*).



Supplemental Figure 3.2. Original DNA gels examining expression of *PpISA1*, *PpISA2* or *PpActin* via semi-quantitative RT-PCR in the wildtype (WT) or mutant experimental lines. λ PstI is λ phage DNA digested with *PstI.*



Supplemental Figure 3.3. Original SDS-PAGE gels showing purified recombinant His-Tagged PpISA1 and PpISA2 proteins. PageRuler molecular weight marker is shown in Lane1.

Chapter Four

Final discussions, recommendations and conclusions

4.1. Introduction

The overall aim of this study was to gain a better understanding of the function of some of the genes known to play a role in angiosperms starch metabolism in the Bryophyte *Physcomitrella patens.* As described in chapter 1, this plant is well suited as a model organism as it possesses many of the structures found in vascular plants, even though Bryophytes had diverged from seed plants more than 400 million years ago. The great potential of this plant in unravelling the basic molecular mechanisms that have been conserved throughout the evolution of land plants is of great importance. Because starch is a valuable industrial commodity, the fundamental mechanisms involved in its metabolism have been extensively studied in several plants, most especially *Arabidopsis*. Elucidating the mechanisms responsible for the production and breakdown of starch in *Physcomitrella,* would allow us to study to what extent key enzymes involved in these processes are conserved between mosses and vascular plants. This project set out to examine two aspects of starch metabolism in plants: the roles of glucan, water dikinase (GWD) and isoamylase (ISA) in the degradation and biosynthesis of starch. In particular, this study focused on two specific ISA's (ISA1 and ISA2) and two GWD's (GWDa and GWDb). To fulfil this, I generated two sets of single and double mutants for both GWD and ISA by taking advantage of *Physcomitrella*'s highly efficient native homologous recombination that makes gene targeting feasible (Cove, 2005).

The first experimental chapter (Chapter 2) sheds light on the importance of GWD in starch degradation and gametophore development, while the second experimental chapter (Chapter 3) examines whether PpISA1 and PpISA2 play the same role during the biosynthesis of starch as in vascular plants and *Chlamydomonas reinhardtii*. This chapter will reflect on the broader implication of these, to address the study's research questions. Furthermore, interesting future lines of research will be briefly discussed.

4.2 Examination of starch degradation in *Physcomitrella patens*

4.2.1 *Ppgwda* single and *Ppgwda/Ppgwdb* double mutant plants demonstrate reduced starch degradation and increased starch accumulation

Research examining GWD in vascular plants has provided an understanding of the importance of photosynthetic starch degradation on the growth and development of plants. Transient starch that accumulates during the day as a result of excess carbon fixation via photosynthesis is important for plant growth. During the night, when photosynthesis is not possible, it is degraded to provide a supply of sugars that sustains plant metabolism and growth (Cheng et al., 1998; Zeeman and Rees, 1999; Sulpice et al., 2009). Starch degradation maintains adequate sugar levels throughout the night and that sugar levels decrease when starch is exhausted (Gibon et al., 2004).

The rate of starch degradation in *Arabidopsis thaliana* leaves has been reported to occur at a constant rate during the night to the extent that about 95% of all starch is utilized by dawn. Many experiments have shown that this pattern of utilization is important for normal plant growth. If this process is disturbed by environmental conditions (such as artificial extension of the night beyond the normal dawn) or mutations affecting starch metabolism, the plants demonstrate symptoms of carbon starvation and reduced growth rate (Gibon et al., 2004; Smith and Stitt, 2007; Usadel et al., 2008; Graf et al., 2010; Pantin et al., 2011; Yazdanbakhsh et al., 2011).

A number of studies have shown that the pattern of starch dynamics undergoes long-term changes which enable plants to adapt to short- and long-day conditions. For example, if day length becomes progressively shorter, starch is synthesized at a faster rate during the light period and is degraded more slowly at night (Gibon et al., 2004). Remarkably, the rate of starch degradation is also immediately adjusted in response to unexpected environmental alterations. When plants are subjected to a sudden early or late onset of night, starch degradation rates are altered to ensure that carbohydrate reserves last until the next dawn (Graf et al., 2010; Scialdone et al., 2013). Equally, changes in light intensity that alter the amount of starch synthesised is immediately compensated for by an appropriate change in the rate of night-time starch degradation (Scialdone et al., 2013).

The circadian clock plays a critical role in the regulation of starch degradation. Mutations in circadian clock genes lead to alterations in the normal turnover of starch (Bernier et al., 1993; Corbersier et al., 1998; Graf et al., 2010; Stitt and Zeeman, 2012). For instance, the combined mutations in two transcription factors (*LATE ELONGATED HYPOCOTYL* (*LHY)* and *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* lead to a short circadian clock rhythm and premature exhaustion of starch reserves at night. This is accompanied by depletion of sugars and an inhibition of root growth (Graf et al., 2010).

Starch degradation has been extensively studied in *Arabidopsis*-with mutants inhibiting transient starch degradation in leaves resulting in lines displaying a starch excess (SEX) phenotype. Although some research has been performed in *Chlamydomonas* using a forward genetic approach, to identify mutants affecting starch catabolism, there is still a need to expand our knowledge, and acquire a deeper understanding of this process in non-vascular plants, including Bryophytes.

In the chapter 2, I decided to establish the function of GWD in *P. patens,* to observe how its mutation would affect its growth and development. This was to examine whether mutations affecting this enzyme would have similar effects to those found in *Arabidopsis gwd1*/*sex1* mutants.

Phylogenetic analysis demonstrated that a recent gene duplication resulted in five GWD isoforms. Two of these (*PpGWDa* and *PpGWDb*) were most similar to *GWD1* or *GWD2* genes from angiosperms. Analysis of both single and double mutant lines lacking putative GWD1/GWD2 isoforms demonstrated reduced amounts of starch phosphate, confirming that both isoforms demonstrate GWD-like function (Lorberth et al., 1998; Blennow et al., 1999; Yu et al., 2001; Kötting et al., 2005). In vascular plants it is thought that phosphorylation of the glucosyl moieties by GWD disrupts the starch's double helical structure, making it accessible to iso- and β-amylases that degrade surface glucans (Ritte et al., 2006; Blennow and Engelsen, 2010). Phosphate groups are subsequently removed in a series of reactions that involve participation by the phosphatases SEX4 and LSF2, as β-amylases cannot bypass the phosphate groups (Hejazi et al., 2010; Santelia et al., 2011; Meekins et al 2015).

I could further show that starch in *Physcomitrella* is synthesized during the day and degraded at night. Interestingly, the *Ppgwda* single mutant and *Ppgwda*/*Ppgwdb* double mutant (DM) degraded more starch than either the *Ppgwdb* single mutant or the wild type (WT). This was attributed to the higher amounts of starch present

in photosynthetic tissue at the start of the experiment caused by the small amount of starch that was mobilized during the night.

Analysis of the plants demonstrated that mutations in *PpGWDa* led to plants with an inability to develop gametophores. These structures are important for *P. patens* as they are involved in gametogenesis as well as the formation of diploid sporophytes which form haploid spores. This provides a competitive advantage for *Physcomitrella* as it allows both sexual reproduction, as well as the ability of spores to spread to new environments and to survive unfavourable conditions. We hypothesized that this phenotype was due to the observed low soluble sugar levels that would affect normal development and growth of gametophores. This hypothesis is supported by the observation that the mutants developed gametophores when grown on media supplemented with glucose suggesting that the alteration in gametophore development is caused by low soluble sugars. Clearly, any alteration in metabolism within *P. patens* that eliminated gametophores would have profound implications for survival of this plant as it would be unable to produce spores. Taken together, these results show that *PpGWDa* has distinct physiological roles in *P. patens* and that starch derived soluble sugars influence the production of gametophores. It is interesting to compare this phenotype with angiosperm mutants affected in starch metabolism. *Arabidopsis sex1* (Yu et al., 2001), *sex4* (Zeeman et al., 1998), *bam3* (Lao et al., 1999) and *gi* (Eimert et al., 1995) mutations all alter the rate of starch biosynthesis or degradation, and all display late flowering phenotypes. This indicates that alterations in carbon metabolism affect development of gametogenesis forming organs and that this link developed very early in land plant evolution.

There are several possible mechanisms that could lead to the inhibition of gametophore development. For instance, Goss et al. (2012) demonstrated that mutations in a cellulose synthase gene (*PpCESA 5*) resulted in severe cellulose deficiency due to a reduction in the amount of carbon skeletons supplied as a substrate for production of cellulose in *P. patens*. Gametophore buds in these mutants failed to develop into normal leafy gametophores suggesting the importance of cellulose in the development of gametophores. This could be investigated by measuring cellulose amounts in the *Ppgwd* mutants. Secondly, there could be downstream effects on hormone amounts or expression of genes and miRNA's that influence gametophore initiation. RNAseq experiments examining alterations in gene expression in the *Ppgwd* mutants alongside phytohormone analysis could be used to examine this. Analysis of growth on media

containing different phytohormones could also elucidate their involvement. Thirdly, various sugar sensing mechanisms could come into play. Genes known to be involved in sugar sensing in Angiosperms, such as hexokinase and SnRK affect *Physcomitrella* growth. According to Olsson et al. (2003), the hexokinase *PpHXK1* phosphorylates glucose within chloroplasts and has been reported to be the major glucose phosphorylating enzyme in *P. patens*. Plants deficient in PpHXK1 demonstrate a complex phenotype that includes failure to develop proper gametophores. As with *Ppgwda*, growth of *Pphxk1* mutants on glucose containing medium enhances gametophore number. Similarly, double knockout mutants in two SnRK like genes (*PpSNF1* and *PpSNF2*; Thelander et al., 2004) produce gametophores that are smaller than the wild-type control. Based on these data, it would be interesting to generate a double knockout of the *PpGWDa* and *PpHXK1* or *PpSNF* genes and analyse how these impact growth and development. Finally, an unknown mechanism affecting gametophore development could be involved. This could be examined through chemical mutagenesis of the *Ppgwda* mutant and analysis of suppression mutants that regain the ability to develop gametophores.

4.2.2 *Ppgwdb* mutations affects starch phosphorylation and gametophore number, but not starch amounts

Mutations in *Ppgwda* affected starch catabolism and eliminated gametophore development. On the other hand, mutations in *Ppgwdb* reduced starch phosphate and gametophore numbers, but did not affect starch or soluble sugar amounts. Clearly there is some functional redundancy between the two isoforms, as demonstrated by the almost complete elimination of phosphate from starch in the *Ppgwda*/*Ppgwdb* double mutants. There are two possible scenarios that can explain this. The first is that PpGWDa and PpGWDB exist in the same cells within *Physcomitrella* and phosphorylate starch together within the chloroplast. The second is that they are expressed in spatially distinct cell types where PpGWDa is present in cells that contain the majority of starch with the plant. This second hypothesis could help to explain the reduction in gametophore numbers in *Ppgwdb* mutants if PpGWDb is present in cells that lead to induction of a small proportion of the total number of gametophores, but which accumulate only a small amount of the total starch in the plant. Fusions between promoters from *PpGWDa* and *PpGWDb* with reporter genes could show expression patterns and potentially identify how the mutation in *PpGWDb* affects gametophore number.

4.2.3 Expanding the understanding of starch degradation in non-vascular plants

A comparative bioinformatics study of green algal genomes has demonstrated the presence of at least one isoform for each of the steps involved in starch metabolism (Deschamps et al., 2008) which indicates that the pathway established in angiosperms may well be similar in non-vascular plants. However, the presence of a gene does not demonstrate functionality and data examining mutants in those genes is needed to confirm this. Such studies are just starting in two non-vascular plants, *Chlamydomonas* (Tuncay et al., 2013; Jang et al., 205; Findinier et al., 2017; 2019) and, in this dissertation, *Physcomitrella patens*. A screen in the unicellular green algae, *Chlamydomonas* reinhardtii, identified a β-amylase and a plastidial maltose transporter (MEX) as being involved in starch degradation (Tuncay et al., 2013). Recently, a mutant (*bsg1*) defective in starch degradation was also identified in this green alga, which contains a lesion in a gene most similar to phosphoglucan water dikinases (Findinier et al., 2019).

In chapter 3, I show that GWD affects starch phosphorylation and catabolism in *P. patens* consistent with the model of starch degradation established in *Arabidopsis*. Clearly more research needs to be performed to demonstrate if the rest of this pathway is also conserved in *Physcomitrella*. Recently it has been demonstrated that part of that pathway differs in green algae, where it has been found that the main sugar exported from the plastid to cytosol is glucose not maltose (Findinier et al., 2017). It would, therefore, be of interest to examine what sugar is transported into the cytosol in *P. patens*. Such analysis can be challenging in *Physcomitrella* because of the large number of genes involved in metabolism, for example there are four MEX type transporters present in the *Physcomitrella* genome (Chapter 2) - nevertheless, it would be of interest to examine these. More broadly, an understanding of starch degradation in red and brown algae would be of great interest as it would allow an understanding of this pathway in more distantly related plant species.

4.3 Isoamylases and starch formation in *P. patens*

4.3.1 Both PpISA1 and PpISA2 mutations lead to accumulation of water-soluble content

The second experimental chapter (Chapter 3) investigated the role of ISA in moss *Physcomitrella patens.* The major objective was to identify and characterize ISA to

determine if they are involved in *P. patens* starch biosynthesis. As outlined in Chapter 1, ISA1 and ISA2 are members of the glycoside hydrolase family, and they play a role in the hydrolysis of α-1, 6 glycosidic linkages in amylopectin. Current evidence suggests that ISA1 and ISA2 are present as heteromultimeric enzyme complexes in all plant species studied (Fujita et al., 1999; Hussain et al., 2003; Utsumi and Nakamura, 2006; Facon et al., 2013; Sundberg et al., 2013; Streb and Zeeman, 2014), with ISA1 serving as the active protein and ISA2 a non-catalytic subunit (Macgregor, 1993; Hussain et al., 2003; Sundberg et al., 2013). In these species, the heteromultimeric complex was found to be crucial for amylopectin biosynthesis (Hussain et al., 2003; Bustos et al., 2004; Delatte et al., 2005; Wattebled et al., 2005; 2008) and loss of either ISA1 or ISA2 results in reduced starch content and an accumulation of a water-soluble polysaccharide (WSP) called phytoglycogen (James et al., 1995; Nakamura et al., 1996; Dinges et al., 2001; Burton et al., 2002). However, cereal species and *Chlamydomonas reinhardtii* also possess a homomeric enzyme complex with ISA1 present as a dimer (Kubo et al., 2010; Sim et al., 2014) where loss of ISA2 leads to accumulation of either no, or reduced amounts of phytoglycogen due to the presence of the active ISA1 homomeric complex (Fujita et al. 1999; Dauvillée et al., 2001; Kubo et al., 2010; Utsumi et al., 2011; Facon et al. 2013; Sim et al., 2014). Furthermore, examination of recombinant maize ISA1 alone by Facon et al. (2013) in *E. coli* demonstrated activity in zymograms, whereas both ISA1 and ISA2 had to be present in *Arabidopsis* and potato before significant activity was observed. These studies support the idea that ISA2 is not absolutely required for activity in cereals, while it is an important factor in dicotyledonous plants (Delatte et al., 2005; Wattebled et al., 2005; Hussain et al., 2003; Kubo et al., 2010; Lin et al 2013; Utsumi et al 2011).

In Chapter 3 isoamylase activity was assessed in *P. patens* using activity gels where crude plant extracts were separated. Mutations in either *PpISA1*, *PpISA2* or both led to the loss of one activity band. This suggests that, similar to dicotyledonous plants the two isoforms exist in a heterocomplex which is unstable when either protein partner is lost. This demonstrates that *P. patens* requires both ISA1 and ISA2 for normal starch synthesis.

Interestingly I examined the amount of glucose 6-phosphate in WSP from each mutant and showed significant increases in both *Ppisa1* and double mutant lines. The phosphate is presumable incorporated by GWD isoforms, but the question arises as to why it is not present in large amounts in WSP from *Ppisa2* mutants. It

has been demonstrated that GWD preferentially phosphorylates specific chain lengths (Mikkelsen et al., 2004). If chain lengths in the phytoglycogen of the *Ppgwda* and *Ppgwdb* mutants differ, then this could help explain the different amounts of covalently bound phosphate. Analysis of the chain length distributions of the different WSP's alongside incubation experiments with recombinant PpGWD will help to elucidate this. No data on WSP bound phosphate is present in the literature, so it is unclear if it is present in other plant species. It is also unclear what its function is? This could be examined through analysis of *Ppisa1*/*Ppgwda*/*Ppgwdb* triple mutants to examine what occurs when the WSP is not phosphorylated.

4.3.2 Assays demonstrate that PpISA2 is an active protein, so what's next?

In my experiments, purified recombinant PpISA2 protein demonstrated activity. Despite the lack of observable contaminating polypeptides in the purified enzymes, we cannot rule out the possibility that the native *E. coli* debranching enzyme co-purified with PpISA2. To demonstrate if this is the case or not, polypeptides present in the recombinant proteins sample could be identified by peptide fingerprinting.

On the assumption that PpISA2 is active, it opens up interesting possibilities for examination of structure function relationships in ISA polypeptides. Eight amino acid residues within the ISA active site are believed to be essential for catalysis (Hussain et al. 2003). The inactivity of *Arabidopsis*, maize and rice ISA2 are thought to be due to substitutions of six of these eight amino acids, with two other residues (Val-379 and His-382) remaining conserved. My data would imply that the substitutions of R450V, D452V, E527D, H619N and D620S identified by Hussain et al. (2003) in ISA2 are unimportant for ISA activity as they are all found in PpISA2. One amino acid (Asp-377) altered in the active site of Angiosperm ISA2 isoforms is, however found in PpISA2. It is conceivable that this amino acid is essential for ISA activity. This could be tested through site directed mutagenesis of Asp-377 in PpISA2 where would be altered to the same amino acid found in angiosperm ISA2's, or the reverse experiment where an angiosperm ISA2 is mutated so that it contains Asp-377. Activity of ISA2 has only been examined in angiosperm proteins. My data also indicates that it would be worthwhile to determine ISA2 activity from other plant species, including green algae, other bryophytes and basal land plants.

Finally, it would be useful to express both PpISA1 and/or PpISA2 in the *Arabidopsis isa1/isa2* single or double mutants to examine if either of both *Physcomitrella* proteins can complement the mutations. In addition, a yeast system has recently been established where the starch pathway was recreated (Pfister et al., 2016). In that study it was shown that isoamylase activity was essential for the formation of crystalline polyglucan. The expression of PpISA1 and/or PpISA2 in such a system could be used to examine their roles in starch metabolism.

4.3.3 Starch metabolism: An important factor in the development and growth of P. patens

Analysis of both single and double mutant ISA lines demonstrated significant differences compared to the wild type. In chapter 3, I described that these mutant lines exhibited a slow growth phenomenon, and as a result, gametophores took longer to develop and were also fewer in number compared to the wild type. This observation suggested that, the disruption of ISA leads to a slow transition from protonemata to gametophores. Additionally, gametophore length was severely reduced especially in double mutants. This suggests a role for ISA in the development of gametophores. The reasons for this are unclear. Perhaps the accumulation of a water-soluble polysaccharide compared with insoluble starch leads to alterations in osmotic potential with cells? This might affect cell expansion. Nevertheless, the reduction in gametophore number in the *PpISA* mutants alongside their elimination in *Ppgwda* mutants indicates a general link between starch metabolism and gametophore development.

4.4 Outlook

This project aimed to identify and characterize enzymes in *Physcomitrella* to examine if they are involved in *P. patens* starch metabolism. Although, these genes have been extensively studied in starch metabolism in different species, this study was the first time that GWD and ISA function was described in a bryophyte. Overall, my results and those of others (Olsson et al.,2003; Thelander et al., 2004; Nillson et al.,2011; Goss et al., 2012) have demonstrated that this tiny plant is an excellent plant model for studying carbon metabolism. Specifically, the data I presented in chapter 2 and 3 add to the body of work and indicate that there are no major

differences in the functional roles of GWD and ISA enzymes in *Physcomitrella* compared with angiosperms.

Although this work highlights the importance of starch metabolism in development and growth of *P. patens*, there is still more to be done to address the knowledge gap that exists around this pathway in non-vascular plants. For example, as discussed in chapter 1, ISA3 and LDA play a role in the biosynthesis of starch and are also known to be playing a role in starch degradation in angiosperms. Interestingly, loss of ISA3 in *Arabidopsis* leaves also leads to a starch excess phenotype, and a double mutation lacking both ISA3 and LDA results in an even a more severe phenotype (Wattebled et al., 2005). Taking this further, an eventual scientific goal would be to generate mutant lines where these genes (ISA3 and LDA) are knocked out individually, simultaneously and in combination with GWD1A, to help us understand how they act together to control starch degradation and prevent the starch excess phenotype.

Amylopectin biosynthesis has proven to be a highly complex process in plants. A vital aspect in the presence of multiple enzyme within protein complexes. Experiments conducted using maize and wheat endosperms have confirmed the presence of multienzyme complexes containing starch synthases and branching enzyme isoforms (Tetlow et al., 2004; 2008; Hennen-Bierwagen et al., 2008; 2009; Lin et al. 2012). The formation of some of these complexes is suggested to be important for the synthesis of proper amylopectin. It would be interesting to assess the formation of protein complexes containing starch metabolic enzymes in *P. patens* to examine if they form and how they influence starch formation.

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